Henry Liu
Alan D. Kaye
Jonathan S. Jahr
Editors

Blood Substitutes and Oxygen Biotherapeutics

Springer

Henry Liu • Alan D. Kaye • Jonathan S. Jahr
Editors

Blood Substitutes and Oxygen Biotherapeutics

Springer

Editors
Henry Liu
Anesthesiology & Critical Care
University of Pennsylvania
Philadelphia, PA, USA

Jonathan S. Jahr
Department of Anesthesiology and
Perioperative Medicine
David Geffen School of Medicine at UCLA
Los Angeles, CA, USA

Alan D. Kaye
Department of Anesthesiology
and Department of Pharmacology
Toxicology, and Neurosciences
LSU School of Medicine
Shreveport, LA, USA

Department of Anesthesiology and Pharmacology
LSU School of Medicine
New Orleans, LA, USA

Department of Anesthesiology and Pharmacology
Tulane School of Medicine
New Orleans, LA, USA

ISBN 978-3-030-95974-6 ISBN 978-3-030-95975-3 (eBook)
https://doi.org/10.1007/978-3-030-95975-3

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Many thanks to all my mentors who taught me how to become a good physician during the years of my training, especially Dr. M. Jane Matjasko, former president of ABA and former chair of the Department of Anesthesiology, University of Maryland Medical Center, who encouraged me to pursue an academic career. My thanks also go to my residents, students, trainees, and patients who inspired me to write this book and to be a better educator. Most importantly, I thank my family, my wife Marilyn Li, MD, my daughter Cindy Liu, and my son Geoffrey Liu, MD; their support and encouragement are critical to every bit of my success.

Henry Liu, MD, MS, DABA, FASA
Professor of Anesthesiology & Critical Care Medicine
Perelman School of Medicine at the University of Pennsylvania
3400 Spruce Street
Philadelphia, PA 19104

To my wife Dr. Kim Kaye for being the best spouse a man could ask for in his life.
Also, my mother Florence Feldman who taught me to accomplish tasks with the highest of quality, and to reach my dreams and goals in life. To my brother Dr. Adam M. Kaye, Pharm D, for his loving support, acts of kindness, and help over the past 50 plus years.
Finally, to all my teachers, trainees, and colleagues for their friendship, support, and collaboration at the University of Arizona in Tucson, Ochsner Clinic in New Orleans, Massachusetts General Hospital/Harvard School of Medicine in Boston, Tulane School of Medicine in New Orleans, Texas Tech Health Sciences Center in Lubbock, LSU School of Medicine in New Orleans, and LSU School of Medicine in Shreveport.

Alan D. Kaye, MD, PhD, DABA, DABPM, DABIPP, FASA
Vice Chancellor of Academic Affairs, Chief Academic Officer, and Provost
Pain Program Fellowship Director
Professor, Department of Anesthesiology and Pharmacology, Toxicology, and Neurosciences
Louisiana State University School of Medicine
1501 Kings Hwy, Shreveport, LA, USA 71103

My thanks go to my entire family for their forbearance with my schedule during the intense work on this textbook and help with computer issues, especially in formatting work. My wishes are to thank Rachel Jahr for her graphic design assistance. I would like to express my gratitude sincerely to all the colleagues, students, graduates, residents, and postdoctoral fellows with whom I have had the honor to work all these years, to help me to accomplish meaningful goals and, hopefully, enable many patients to survive serious injury/operations and allow them to experience less pain.

Jonathan S. Jahr, MD, PhD, DABA, FASA
Professor Emeritus of Anesthesiology
David Geffen School of Medicine
Ronald Reagan UCLA Medical Center

Foreword

Blood Substitutes and Oxygen Biotherapeutics

Is the Final Destination in the Long, Winding, Bumpy Road Finally in Sight?

It is a tremendous honor to be invited to offer some introductory comments to this comprehensive treatise on blood substitutes and oxygen biotherapeutics. I have interacted in the past with many of the chapter authors, and have studied with admiration the offerings of many of the distinguished colleagues who have endeavored with admirable persistence in this important but slowly and erratically developing field. The editors have been diligent in selecting experts who have pursued the ambitious goal of developing new therapies, and the result is a review of where the field has been, moving to discussions of where the opportunities to achieve licensed therapeutic products will need to proceed. Most of us are familiar with the early encouraging work in the field, perhaps epitomized by the often-featured view of a rodent in a beaker filled with a blood substitute that sustained its life. With retrospective naivety, it might have appeared to the readers and probably to me that therapeutic products would become available in a short interval. The many offerings in this book demonstrate our unrequited optimism but hopefully the synthesis demonstrates that the goal remains cogent and that our endeavors have progressed with knowledge and experience gained along the treacherous path.

The editors have provided extensive coverage to blood substitutes in a logical progression through five sections. The first reviews transfusion science and describes the development of transfusion therapies based upon the physiology of blood and in particular hemoglobin. It discusses evolving transfusion therapies with emphasis upon where blood substitutes might meet a continuing unmet medical need. An important chapter reviews the role of nitric oxide, an area of red cell physiology largely unknown during the early stages of blood substitute development, but now recognized to be an impediment to satisfactory clinical products if its involvement in vasoactivity is not addressed.

The second section provides background on pharmacology of oxygen therapeutic through iterations such as perfluorocarbons and hemoglobin-based oxygen carriers. It reviews previous attempts to limit the recognized toxicities of these products through maneuvers such as cross-linking, conjugation, polymerization, and encapsulation, with limited success in addressing the issues of vasoactivity. Although these product modifications supported early clinical trials, they helped to focus on the ultimate design of a potentially successful product, which has enabled our growing knowledge of molecular biology to add more specific compounds to hemoglobin, which will enable oxygen delivery, reduce vasoactivity, and reduce the inflammation that often accompanies the clinical problems being addressed by these agents. The third section builds upon this experience base to describe products in current and past development, building upon the interpretation of the documented failures of these early products. These developing therapies include further manipulation of hemoglobin, novel additives that should increase the efficacy of these products, and strategies to develop therapeutic agents that might include artificial red cells or oxygen carriers from untraditional sources.

Section four describes a number of proposed products in various stages of development. Many of these formulations are now of historical interest, seemingly attractive approaches at

previous times that failed in operational development or more extensive clinical trials. These presentations all emphasize "lessons learned," which will hopefully lead to successful products in the future. Some of these products with substantial persistence have already undergone extensive trial experience and clinical use with evidence of benefit but remain unapproved due to high regulatory hurdles. Others have not yet been definitively tested or failed to be provide approvable results despite early promise in preliminary studies. The fifth and final section discusses specific indications for future studies and highlights the regulatory requirements they must address to achieve clinical availability and commercialization. Proposals are provided that suggest that the goal of these therapies is not to replace red cell transfusions but treat severe anemia cases where transfusion might not be the best option

The offerings of this treatise provide a comprehensive history of previous attempts and failures for blood substitute development and suggest some new ways of thinking that might be helpful to achieve the ultimate goal. There has been considerable discussion about the indications for blood substitutes. In consideration of how these products could be used, the obvious issue is to balance the clinical benefits versus the recognized toxicities. In the early 1980s, blood substitutes generated widespread public enthusiasm as a means to avoid the known infectious risks of AIDS and hepatitis. Regulatory agencies applied understandable caution for approval for this indication, as other means to avoid these transfusion complications became available. It was also appreciated that the perceived benefits were not justified by the toxicities of vasoconstriction and the clearly recognized shortcoming of blood substitutes with transient in vivo survival. On the other hand, most observers now recognize that red cells do not address all medical needs in some instances of acute anemia and that blood substitutes could be lifesaving for patients in whom blood is not available or the safest option. In these cases, the potential benefits would justify the acceptance of some potential adverse effects. Specific examples would include religious objectors who refuse red cell transfusions but often accept blood substitutes as a matter of conscience. Another cadre of patients who would benefit are those who have antibodies due to alloimmunization or autoimmune hemolytic anemia where compatible red cells are difficult to find so that a blood substitute can provide a bridge until the immunohematologic difficulties have been addressed. Other indications were not foreseen by early investigators. A transfusion complication called hyperhemolysis has now been identified, where transfused patients begin to hemolyze the transfused cells and their own autologous red cells and frequently develop profound anemia. This condition can complicate management of patients with sickle cell anemia but has been described with other diagnoses. Continued red cell transfusions in these cases are ineffective and futile, suggesting a temporizing therapy with a blood substitute could be a critical stabilizing therapy. For these indications for patients with severe anemia, it is now appreciated that clinical trials with randomization and blinding are difficult to develop and have ethical challenges. It is also becoming clear that the study target and the ultimate goal of therapy are not red cell transfusion replacement or avoidance but preventing the ravages of life-threatening severe anemia.

Patients with severe hemorrhage in military or civilian settings have long been a prime candidate for blood substitute implementation. Much of the clinical investigation with blood substitutes has targeted trauma, in part due to the size of the potential market. Clinical trials in this area have been difficult, however, because of the distant settings for the military and many civilian cases with limited access to the necessary measures in these complicated studies. In a large trial of blood substitutes in civilians, the transit time to acute trauma centers was too short to allow sufficient infusions of blood substitutes. In recent studies of plasma infusions in trauma settings, it is interesting that benefit was shown in patients with long transport times without benefit in a centralized trauma program with short transit times; studies of blood substitutes in patients with long transit times might have shown different outcomes but are difficult to perform. Another complication of trauma studies is the evolution of therapy that is occurring while trials of blood substitutes are performed or contemplated. Early approaches to trauma emphasized fluid resuscitation at the site of injury where the patient is stabilized prior to transport. Current programs of damage control resuscitation avoid fluids and tolerate hypotension,

with rapid transport to a trauma center to avoid "popping the clot." The role of blood substitutes in evolving trauma care is the subject of current debate. Although the potential patient population is large and the risk of mortality in these situations is high, it remains a difficult group to study in a controlled clinical trial suitable for regulatory approval.

The editors are to be congratulated for amassing this review of the long and arduous road that has yet to provide a licensed blood substitute in the United States. It is anticipated that even skeptics who disparage the need for these therapies will recognize that toxicities can be overcome, and a small group of patients with currently accepted indications will benefit in the near future. It is also possible that continued development may expand the utility of biotherapeutics to other patient groups such as solid organ transplant recipients and patients with uncontrolled inflammatory states. This book, recognizing the failures of the past but providing insights into paths to move forward, is an important contribution to the impetus to continue this important work.

Paul M. Ness
Professor, Pathology, Medicine, and Oncology
Johns Hopkins University School of Medicine
Baltimore, MD, USA

Contents

Contributors

Abe Abuchowski Prolong, Inc., South Plainfield, NJ, USA

Seetharama Acharya Departments of Medicine, Physiology and Biophysics, and of Radiology Albert Einstein College of Medicine, Bronx, NY, USA

Department of Bioengineering, UCSD, San Diego, CA, USA

Abdu I. Alayash Laboratory of Biochemistry and Vascular Biology, Center for Biologics Evaluation and Research, Food and Drug Administration (FDA), Silver Spring, MD, USA

Ahmad Alli St. Michael's Hospital, Department of Anesthesiology and Pain Medicine, University of Toronto, Toronto, ON, Canada

Hiroshi Azuma Department of Pediatrics, Asahikawa Medical University, Asahikawa, Japan

Hans Bäumler Institute of Transfusion Medicine, Charité-Universitätsmedizin Berlin, Berlin, Germany

George P. Biro University of Ottawa, Ottawa, ON, Canada

Joanne M. Blanckenberg Netcare Milpark Hospital, Parktown, Johannesburg, South Africa

Craig Branch Departments of Medicine, Physiology and Biophysics, and of Radiology Albert Einstein College of Medicine, Bronx, NY, USA

Department of Bioengineering, UCSD, San Diego, CA, USA

Kristin Brennan Department of Anesthesiology and Perioperative Medicine, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, PA, USA

Meghan Brennan University of Florida College of Medicine, Department of Anesthesiology, Gainesville, Florida, USA

Mary Brummet Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

Center for Blood Oxygen Transport and Hemostasis (CBOTH), University of Maryland School of Medicine, Baltimore, MD, USA

Paul Buehler Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

Center for Blood Oxygen Transport and Hemostasis (CBOTH), University of Maryland School of Medicine, Baltimore, MD, USA

Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, USA

Yll Buqa Dental Medicine, University Dentistry Clinical Center of Kosovo, Pristina, Republic of Kosovo

Kenneth Burhop Fallbrook, CA, USA

Thomas Ming Swi Chang Artificial Cells and Organs Research Centre, Departments of Physiology, Medicine and Biomedical Engineering, Faculty of Medicine, McGill University, Montreal, QC, Canada

Peter C. Y. Chen Institute for Biomedical Sciences, San Diego, CA, USA

Department of Bioengineering, University of California, San Diego, La Jolla, CA, USA

Davy C. H. Cheng Department of Anesthesia & Perioperative Medicine, Schulich School of Medicine, Western Ontario University, London, ON, Canada

School of Medicine, The Chinese University of Hong Kong, Shenzhen, China

Verghese T. Cherian Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, PA, USA

Anthony T. W. Cheung Department of Pathology and Laboratory Medicine, University of California, Davis School of Medicine, Sacramento, CA, USA

Institute for Biomedical Sciences, San Diego, CA, USA

Jacob Cole Department of Anesthesiology, Naval Medical Center Portsmouth, Portsmouth, VA, USA

Steven A. Conrad Departments of Medicine, Emergency Medicine, Pediatrics and Surgery, Louisiana State University Health Shreveport, Shreveport, LA, USA

Elyse M. Cornett Department of Anesthesiology, LSU Health Sciences Center, Shreveport, Shreveport, LA, USA

Department of Anesthesiology, LSU Health Shreveport, Shreveport, LA, USA

Alexis Cralley Ernest E Moore Shock Trauma Center at Denver Health, Denver, CO, USA

Department of Surgery, University of Colorado Denver, Aurora, CO, USA

Rageev Dalal Department of Anesthesiology and Perioperative Medicine, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, PA, USA

William Davis NYU-Grossman School of Medicine, Department of Anesthesiology, New York, NY, USA

William G. Day Department of Internal Medicine, Naval Medical Center Portsmouth, Portsmouth, VA, USA

Joe De Angelo Platelet Therapeutics, LLC, Chapel Hill, NC, USA

Eric Delpy HEMARINA SA – Aéropôle centre, Morlaix, France

Allan Doctor Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

Center for Blood Oxygen Transport and Hemostasis (CBOTH), University of Maryland School of Medicine, Baltimore, MD, USA

University of Maryland School of Medicine, Health Sciences Facility (HSF) III, Baltimore, MD, USA

Aleksander Dokollari CARIM, School for cardiovascular diseases, Department of Cardiac Surgery, University of Maastricht, Maastricht, The Netherlands

Chancellor Donald Tulane University School of Medicine, Department of Medicine, Division of Hematology/Medical Oncology, New Orleans, LA, USA

Shannon Dougherty KaloCyte, Inc., Baltimore, MA, USA

Sean Dowd Department of Chemical and Biological Engineering, Villanova University, Villanova, PA, USA

Jacob Elmer Department of Chemical and Biological Engineering, Villanova University, Villanova, PA, USA

Timothy N. Estep Chart Biotech Consulting, LLC, Erie, CO, USA

Paulo A. Fontes LyGenesis Inc., Pittsburgh, PA, USA

Amanda Frantz University of Florida College of Medicine, Department of Anesthesiology, Gainesville, Florida, USA

Radostina Georgieva Institute of Transfusion Medicine, Charité-Universitätsmedizin Berlin, Berlin, Germany

Department of Medical Physics, Biophysics and Radiology, Medical Faculty, Trakia University, Stara Zagora, Bulgaria

Matthew Hammer Department of Anesthesiology, Mayo Clinic Arizona, Phoenix, AZ, USA

Christopher Ryan Hoffman Thomas Jefferson University Hospital, Philadelphia, PA, USA

Carleton J. C. Hsia AntiRadical Therapeutics, LLC, Sioux Falls, SD, USA

AntiRadical Therapeutics Canada Inc., Rosseau, ON, Canada

Alexander Huynh Thomas Jefferson University Hospital, Philadelphia, PA, USA

Marcos Intaglietta Department of Bioengineering, UCSD, San Diego, CA, USA

Jonathan S. Jahr Department of Anesthesiology and Perioperative Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Mazyar Javidroozi Department of Anesthesiology, Critical Care and Hyperbaric Medicine, Englewood Hospital and Medical Center, Englewood, NJ, USA

Preya Jhita Stanford University, School of Medicine, Stanford, CA, USA

Marc J. Kahn Kirk Kerkorian School of Medicine, University of Nevada Las Vegas, Office of the Dean, Las Vegas, NV, USA

Alan D. Kaye Department of Anesthesiology and Department of Pharmacology, Toxicology, and Neurosciences, LSU School of Medicine, Shreveport, LA, USA

Department of Anesthesiology and Pharmacology, LSU School of Medicine, New Orleans, LA, USA

Department of Anesthesiology and Pharmacology, Tulane School of Medicine, New Orleans, LA, USA

Peter E. Keipert KEIPERT Corp. Life Sciences Consulting, San Diego, CA, USA

Christopher G. Kevil LSU Health Shreveport, Shreveport, LA, USA

Hae Won Kim Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, School of Medicine, Providence, RI, USA

Naoko Kobayashi Department of Chemistry, Nara Medical University, Kashihara, Japan

Tomoko Kure Department of Chemistry, Nara Medical University, Kashihara, Japan

Gary W. Latson Neurosurgical Anesthesiology, Baylor Scott and White Temple Memorial Hospital, Temple, TX, USA

Texas A&M University College of Medicine, Bryan, TX, USA

William Rick Light VirTech Bio, Natick, MA, USA

Jennifer C. Lim Boston College, Chestnut Hill, MA, USA

Andrew H. Lin Department of Cardiology, Naval Medical Center Portsmouth, Portsmouth, VA, USA

Henry Liu Anesthesiology & Critical Care, University of Pennsylvania, Philadelphia, PA, USA

Richard T. Mahon Undersea Medicine Department, Naval Medical Research Center, Silver Spring, MD, USA

C. David Mazer Li Ka Shing Knowledge Institute of St Michael's Hospital, Departments of Anesthesiology Pain Medicine and Physiology, University of Toronto, Toronto, ON, Canada

Department of Anesthesia, Unity Health Toronto, Toronto, ON, Canada

Patrick McQuillan Department of Anesthesiology and Perioperative Medicine, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, PA, USA

Benjamin C. Miller Louisiana State University Health Shreveport, Shreveport, LA, USA

Sumitra Miriyala Department of Cellular Biology and Anatomy, Louisiana State University Health Shreveport, Shreveport, LA, USA

Nivesh Mittal KaloCyte, Inc., Baltimore, MA, USA

Parikshit Moitra Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

Center for Blood Oxygen Transport and Hemostasis (CBOTH), University of Maryland School of Medicine, Baltimore, MD, USA

Department of Diagnostic Radiology, University of Maryland School of Medicine, Baltimore, MD, USA

Ernest Moore Ernest E Moore Shock Trauma Center at Denver Health, Denver, CO, USA

Department of Surgery, University of Colorado Denver, Aurora, CO, USA

Sherri Ozawa Institute for Patient Blood Management and Bloodless Medicine and Surgery, Englewood Health, Englewood, NJ, USA

Dipanjan Pan Department of Chemical, Biochemical and Environmental Engineering and Department of Computer Science and Electrical Engineering, University of Maryland Baltimore County, Baltimore, MD, USA

Agya B. A. Prempeh Department of Anesthesia & Perioperative Medicine, London Health Sciences Centre, Western University, London, ON, Canada

Christopher Priavalle Apex Bioscience, Inc., Durham, NC, USA

Stephen Rogers Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

Center for Blood Oxygen Transport and Hemostasis (CBOTH), University of Maryland School of Medicine, Baltimore, MD, USA

Kimia Roghani University of Queensland-Ochsner Clinical School, New Orleans, LA, USA

Allen Rojhani Drexel University, College of Medicine, Philadelphia, PA, USA

Bryan T. Romito Department of Anesthesiology and Pain Management, University of Texas Southwestern Medical Center, Dallas, TX, USA

Jia W. Romito Departments of Anesthesiology and Pain Management, Neurological Surgery, and Neurology, UT Southwestern Medical Center, Dallas, TX, USA

Hiromi Sakai Department of Chemistry, Nara Medical University, Kashihara, Japan

Karla Samaniego Department of Surgery, Texas Tech University Health Sciences Center, School of Medicine, Lubbock, TX, USA

Corey S. Scher NYU-Grossman School of Medicine, Department of Anesthesiology, New York, NY, USA

Martin A. Schreiber Department of Surgery, Division of Trauma and Acute Care Surgery, Oregon Health & Science University, Portland, OR, USA

Aryeh Shander Department of Anesthesiology, Critical Care and Hyperbaric Medicine, Englewood Hospital and Medical Center, Englewood, NJ, USA

Sahar Shekoohi Department of Anesthesiology, LSU Health Sciences Center, Shreveport, Shreveport, LA, USA

Xinggui Shen Department of Pathology, LSU Health Shreveport, Shreveport, LA, USA

Toby A. Silverman Tunnell Government Services, Biomedical Advanced Research and Development Authority (BARDA), Department of Health and Human Services, Washington, DC, USA

Jan Simoni Texas HemoBioTherapeutics & BioInnovation Center and Texas Tech University Health Sciences Center, Lubbock, TX, USA

AntiRadical Therapeutics, LLC, Sioux Falls, SD, USA

Bohdan J. Soltys AntiRadical Therapeutics, LLC, Sioux Falls, SD, USA

AntiRadical Therapeutics Canada Inc., Rosseau, ON, Canada

Philip Spinella Department of Pediatric Critical Care, Washington University in St Louis, St. Louis, MO, USA

Kenneth Steier Touro College of Osteopathic Medicine, Middletown, NY, USA

Sarayu Subramanian Department of Surgery, Division of Trauma and Acute Care Surgery, Oregon Health & Science University, Portland, OR, USA

Amy G. Tsai Department of Bioengineering, UCSD, San Diego, CA, USA

Arjan van der Plaats XVIVO Perfusion, Groningen, Netherlands

Thomas Verbeek Department of Anesthesiology and Perioperative Medicine, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, PA, USA

Qihong Wang Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

Center for Blood Oxygen Transport and Hemostasis (CBOTH), University of Maryland School of Medicine, Baltimore, MD, USA

Matthew A. Warner Division of Critical Care, Department of Anesthesiology and Perioperative Medicine, Mayo Clinic, Rochester, MN, USA

Jonathan H. Waters The Departments of Anesthesiology and Bioengineering, University of Pittsburgh, and The McGowan Institute for Regenerative Medicine, Pittsburgh, PA, USA

Department of Anesthesiology, Magee Women's Hospital, Pittsburgh, PA, USA

Angela C. Weyand Division of Pediatric Hematology and Oncology, Department of Pediatrics, University of Michigan, Ann Arbor, MI, USA

Hanna Wollocko OXYVITA Inc., Middletown, NY, USA

Touro College of Osteopathic Medicine, Middletown, NY, USA

Jacek Wollocko OXYVITA Inc., Middletown, NY, USA

Yu Xiong Institute of Transfusion Medicine, Charité-Universitätsmedizin Berlin, Berlin, Germany

Soojie Yu Department of Anesthesiology, Mayo Clinic Arizona, Scottsdale, AZ, USA

Franck Zal HEMARINA SA – Aéropôle centre, Morlaix, France

Part I

Transfusion: Science and Practice

Erythrocyte Transfusion: Brief History and Current Practice

1

George P. Biro

Make thick my blood…

Shakespeare: Macbeth, Act 1, scene 5.

Introduction

Since ancient times blood has been thought to possess mystical and healing properties and the notion of changing personal characteristics, by means of a transfusion of blood from another species or person with desirable attributes, has been attempted perhaps many times in the sixteenth century. In the seventeenth century a better appreciation of the circulation of blood and that blood loss from hemorrhage could be reversed by a transfusion. William Harvey's revolutionary experiments and the publication of "*Exercitatio Anatomica De Motu Cordis"* in Frankfurt in 1628 introduced the concept of experimentation and direct observation initiating the scientific approach to medicine. Animal-to-human and human-to-human transfusions followed, the first in 1666 and 1818, respectively. Not all these attempts were successful. A French physician and naturalist was tried for murder after some unsuccessful animal-to-human transfusion attempts. Subsequently, transfusion attempts were prohibited in both England and France [1–3]. In much of the nineteenth century blood transfusion was not accepted as a safe medical procedure, except for the work of James Blundell, a prominent London obstetrician, who recognized that certain circumstances necessitated human transfusions. He developed devices for collecting and administering blood to treat obstetrical hemorrhage and established a donor base. More widespread use of transfusions was hindered by a multitude of "technical" barriers, the absence of methods of sterilizing devices, of appropriate anticoagulation and preservative media. Despite the carnage of the American Civil War and European wars in the second half of the nineteenth century, the use transfusions was insignificant. The introduction of saline infusion in 1884 improved the treatment of hemorrhage and dehydration [1].

The use of transfusions in the early decades of the twentieth century were helped by the discovery of the major blood groups. The outbreak of World War I did not see extensive use of transfusions. With the outbreak of the World war II, transfusions of blood and of plasma and albumin became strategic endeavors [4, 5]. Soldiers in the German SS had their blood group tattooed in their armpits but battlefield transfusions were rare.

The approach taken in this chapter is not a conventional chronological narrative of the history. Rather, it will highlight milestones of the surgical and critical care use of erythrocyte transfusions only and will refer to those as "transfusion". Blood products and components and the technological aspects of blood banking will not be included. The overriding theme in this chapter is dealing with blood as a *scarce and expensive resource* that is handled with a view to *risk management, whereby expected benefits and hazards are balanced.* It must be emphasized that compelling evidence by clinical trials of the benefit of transfusion against its known risks was not available.

Milestones in Erythrocyte Transfusion

Karl Landsteiner and Discovery of Major Blood Groups

The "coming of age" of blood transfusion began with the revolutionary contribution of the Austrian-born, American physician and immunologist, Karl Landsteiner.

G. P. Biro (✉)
University of Ottawa, Ottawa, ON, Canada
e-mail: gbiro@uottawa.ca

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_1

The Early Years of Transfusion: The First Milestone

Karl Landsteiner

Karl Landsteiner (1868–1943) is celebrated for his landmark discovery of the ABO blood groups in 1901 and, together with Alexander S. Wiener, for the discovery of the Rhesus factor in 1937. Landsteiner received the Nobel Prize in Medicine and Physiology in 1930 [6–8] (Fig. 1.1).

These discoveries have made it possible to infuse another person's blood to someone in great need of it. The ABO blood group system was discovered by Landsteiner by testing samples of erythrocytes with the addition of samples of serum from other individuals, using the methods of immunology in which he had been trained. Some serum samples caused the blood cells to clump, or agglutinate, while others did not. By repeated testing, he intuited that there must have been some element, an *antibody* in some serum samples that reacted with an *antigen* on the surface of red blood cells, causing agglutination; whereas the serum of others contained a different antibody that did not react with cells from the same person. And that a person's blood contained the same *type* of antigen on the red cells as the antibody in their serum. He categorized these blood types as A, B and C. Erythrocytes of type A would be agglutinated when mixed with serum from a person having type B antibodies in their serum, but not when mixed with serum from a type A person. Erythrocytes from a person with Type A antigen, when mixed with serum from another person with type A erythrocytes, did not agglutinate. A third type, that he named type C, erythrocytes of a person of either type A or type B, were not agglutinated when mixed with serum from Type B or type A person, either. This third, type C, had neither type A nor type B antibody in their sera. Such a person could receive a blood transfusion from either a Type A or Type B donor. The type C was later renamed Type O (or zero, for the original German word "*Ohne*", without).

Fig. 1.1 Portrait of Karl Landsteiner on an Austrian Postal Service commemorative stamp issued on the 100th anniversary of his birth

Ironically, the revolutionary observation was first reported by Landsteiner in a *footnote* in a paper (1900) on pathologic anatomy, describing the agglutination occurring when blood of one person is in contact with that of another person [7, 9]. The actual description of the discovery of the ABC blood groups was published a year later, in 1901. Landsteiner, at first, did not appreciate the importance of his discovery, writing that "I hope that this will be of some use to mankind" [7].

In 1922 he accepted the invitation by Simon Flexner to join the staff of the Rockefeller Institute where he continued to make major discoveries [8].

The discovery of the Rh, or rhesus factor came about from the case described by Bodner and McKie [10]. The obstetrical patient's physician was Dr. Philip Levine who had been an assistant to Landsteiner for several years.

The patient had a first normal pregnancy, but her second pregnancy ended in the loss of her baby and she suffered a massive hemorrhage. Since both her and her husband had Type O blood, Dr. Levine decided to transfuse her from the husband. To his dismay, she had a violent transfusion reaction. Dr. Levine reasoned that there must have been an alternative blood group type antibody involved in the reaction. It turned out that when the patient's serum was tested against her husband's erythrocytes, agglutination occurred. Moreover, the loss of the baby was due an antigen antibody reaction. The mother's antibody had leaked across the placenta and entered the fetal circulation and caused massive lysis of the fetal erythrocytes which were of a different type inherited from the father. This single case was reported by Philip Levine and Rufus Stetson in 1939 in the Journal of the American Medical Association. They noted the similarity of this first detected case with the then few reported cases of iso-immunization after repeated transfusions [11].

Since the mother's serum caused agglutination of erythrocytes of rhesus monkey, and those of other animal species' erythrocytes, the antibody became known as the rhesus, or Rh factor, subsequently renamed type D antibody. A D-negative mother having a D-positive fetus in her first pregnancy has not yet developed antibodies to the fetus' antigens but will do so when the D-positive fetal

cells leak across the placental barrier during delivery. Subsequent pregnancies may be complicated by the mother's anti-D^+ antibodies entering the fetal circulation. The consequences of the presence of anti- D^+ antibody in the mother and its absence in the fetus, the intrauterine hemolysis, became known as the Hemolytic Disease of the Fetus and Newborn (HDFN) [8].

Landsteiner's many contributions have involved the detection of similar patterns of reactions with rhesus blood. In 1940 he and Wiener immunized rabbits and guinea pigs with erythrocytes of rhesus monkeys. This anti- rhesus (anti-Rh) reacted with 85% of human erythrocytes, indicating the frequency of Rh+ phenotype. It is now known that the type D appellation involves many other different agglutinin subtypes detected by cross matching and phenotyping. After the original discovery of the major blood groups, Landsteiner and coworkers and many followers discovered at least 36 other systems of minor subgroup types with weaker isoreactions [8].

In addition to these important discoveries, Landsteiner also made many others, including the recognition of the viral origin of poliomyelitis, and the diagnostic test for paroxysmal cold hemoglobinuria [6, 7].

Ottenberg (1882–1959) was the first to perform the earliest form of a pretransfusion cross match in 1907, recognizing the clinical significance of avoidance of hemolytic transfusion reactions. This rigorous typing and cross matching have contributed greatly to the safety of early transfusions, however, transfusions remained cumbersome and little used, because of the lack of adequate anticoagulation and storage methods, so that most transfusions were direct donor-to-recipient.

The history of the development of anticoagulant and storage technologies, as well as of those of blood banks, donor bases, and of the introduction of component separation is beyond the scope of this chapter.

The next, second, milestone in this narrative is the recognition that parallel to the risks of anemia, transfusion's benefits may have to be balanced by the recognition that risks are also inherent in transfusions.

Balancing the Risks and Benefits of Anemia and Transfusion: The Second Milestone

The immunologic investigation of blood group types and antigens accelerated in the 1940s as testing technologies improved and became more routine in blood banks. As a result, the use of transfusions of whole blood, and then that of red cell units and components, accelerated, both in cases of acute blood loss (surgery and trauma), and in "chronic "cases (postoperative anemia and in "medical anemia", such as in malignant disease).

With increasing use and availability of blood for transfusion, the prescribing of transfusion became a more common medical treatment where the decision was based on the *expectation of a benefit to the patient* by increasing oxygen carrying capacity and transport. However, there was little objective evidence supporting the expected benefit, especially in the case of single-unit transfusions, that generally result only in a 10 g/L[1] increase in Hb concentration.

Since transfusions had long been in use when the use of clinical trials of establishing efficacy and safety was introduced, transfusion of blood was not subjected to rigorous trials evaluating its efficacy. One of the few medical interventions that remains without rigorous safety and efficacy testing by clinical trials. More recently questions have been raised about when a transfusion is appropriate and the notion of *balancing risks and benefits* of both *the transfusion and of anemia* has become a dominant consideration, but without evidence-based support. The balance is not simple because the transfusion is expected to provide a medical benefit, BUT there are no benefits of severe anemia. On the other hand, both have risks.

The Risks of Anemia

There are no known benefits of severe anemia; its risks need to be considered first.

In an anemic subject oxygen delivery may be impaired, depending on its severity, to an extent that physiological functions may deteriorate, activity may be limited, and organ dysfunction may supervene. This may be explained by the concept of supply dependence when the supply is so limited that a substantial mass of body cells are hypoxic and oxygen consumption falls [12]. There are occasional instances observed when an individual may survive such low hemoglobin concentration[2] as 10-20 g/L. However, retrospective aggregated data from Jehovah's Witnesses who refuse transfusion on religious grounds, reveal how dangerous severe anemia is. At persistent hemoglobin concentration of 11 g/L in-hospital mortality was 100% at 30 days. For every 10 g/L reduction of hemoglobin concentration from 50 g/L, the probability of adverse outcomes, such as myocardial infarction, respiratory and renal failure, etc., doubled [13–15].

Thus, the threat to life represented by severe anemia in compromising oxygen delivery was thought to mandate medical intervention that intended to *prevent,* if possible, such hypoxia (e.g., a case of continuing blood loss). If prevention is not feasible, *amelioration* is required as soon as possible. Thus, a transfusion would be prescribed, in the absence other effective interventions. The expectation of benefit would only be tempered by the then recognized dan-

[1]The international unit of g/L will be used throughout.

[2]Hemoglobin will be abbreviated as Hb; its concentration is abbreviated as [Hb].

ger of *transfusion reactions* (see below). This desired goal is hampered by the lack of objectively definable, universally applicable *thresholds* to facilitate a *rational c*linical decision [12, 16].

The Target Organs of Anemia-Induced Injury

The organs most vulnerable to hypoxia are those of obligate aerobic metabolism, the *brain* and *heart*. Healthy volunteers, subjected to isovolumic hemodilution to a hemoglobin concentration of 50 g/L, exhibited reversible cognitive and memory impairment that was improved by oxygen breathing, indicating the mechanism to be hypoxia [17, 18]. Clinical studies have identified cerebral injury in anemic perioperative patients [16, 19]. Jehovah's Witness patients who refuse transfusion even in the face of severe anemia and/or continuing blood loss (hemoglobin concentration < 80 g/L) have been found in an 11-year review to suffer all-cause mortality rate of 19.8%, and at a hemoglobin concentration ([Hb]) <50 g/L, are very likely to die [15]. These findings strongly suggest that in patients who may have underlying coronary artery disease, severe anemia represents a real threat. In view of the belief that severe anemia is a threat, transfusion had been used in the *expectation of benefit and avoidance of harm.*

An excellent experimental study on rats has shown that anemia induced tissue hypoxia occurs at different levels of [Hb] in different vital organs [20]. The study subjected rats to isovolemic hemodilution to [Hb] concentrations of 90 g/L, or 70 g/L, or 50 g/L and was compared to the baseline of 130 g/L. Tissue hypoxia was indicated by increases in HIF-1α-luciferase[3] activity and NOS[4] expression. Whole body HIF activity increased progressively as the [Hb] was decreased, indicating the presence of tissue hypoxia somewhere in the body even at [Hb] of 90 g/L. In the kidney HIF activity was like baseline at [HB] both at 90 and 70 g/L but became significantly increased at [Hb] = 50 g/L., suggesting a relative degree of tolerance of modest hypoxia. In contrast, the liver exhibited increased HIF expression at [Hb] = 70 g/L, suggesting a higher threshold of hypoxia.

The next, third, milestone in this narrative is the recognition that parallel to the risks of anemia, transfusion's benefits may have to be balanced by the recognition that substantial risks also attend transfusions.

Benefits and Risks of Erythrocyte Transfusion

The Benefits of Transfusion

How do transfusions benefit a patient facing the risks of anemia?

Transfusion is intended to prevent or ameliorate the signs and symptoms of anemia of significant severity that interferes with the supply of oxygen sufficient to the physiological demands of effective functioning. The "physiological benefits" of a two-unit transfusion were described in a study on ICU patients undergoing invasive hemodynamic monitoring [21]. The transfusion's effects included a rise in hematocrit ratio, from 0.22 ± 0.2, to 0.28 ± 0.03, and [Hb], from 76 ± 8 to 94 ± 9 g/L. It is not clear whether the average pre-transfusion [Hb] of 76 g/L would be associated with the need for increased oxygen capacity to ameliorate critical organ hypoxia. There was also significant improvement in hemodynamic variables and oxygen flux and a reduction in the heart rate. However, it is not clear, whether the documented improvements represented a physiologically significant degree of tissue hypoxia or, whether an improvement in blood volume also contributed. The study did not provide definitive evidence of *efficacy*.

A related aspect of transfusion's efficacy is *the timing* of the benefit. Banked erythrocyte units are well documented to have properties different from those of native erythrocyte: the well-known phenomenon of the "*storage lesion*" [22]. This consist of changed biomechanical properties of the erythrocyte that significantly impair perfusion in the microcirculation [23–25]. Animal experiments have shown that the impaired biomechanics of stored erythrocytes' adherence to capillary walls and rigidity represent impaired flow and clinical risk [23, 24, 26]. Finally, the breakdown of cells and the release of their fragments and hemoglobin interfere with NO-mediated vasodilator regulation [22]. These effects are reversible within about 24 hours and the transfused erythrocytes become functional, but their circulating half-life is shortened.

Effectively demonstrating the benefits of transfusion in individual cases is also subject to uncertainties. Not every patient with a given [Hb] is the same as every other patient with same [Hb]. This is due to the variability of individuals' *physiological adaptation* to the anemia that include:

- Duration of anemia: chronic vs acute. Physiological adaptations developed to anemia.
- Increase in cardiac output. Potential redistribution of available blood flow.
- Modification of erythrocytic 2,3 diphospho-glycerate (2,3 DPG) modulating oxygen unloading.
- Presence of comorbidities that may affect or limit the physiological adaptations.

Searching for objective markers of tissue hypoxia lead Hare and colleagues [27] to the kidney as a vulnerable organ during cardio-pulmonary bypass. Acidosis and increased plasma lactate concentration were indicative of some tissue hypoxia. Actual measurements in animal experiments of

[3]Hypoxia Inducible Factor

[4]Nitric Oxide Synthase

renal medullary pO_2 by polarographic electrodes, has shown the presence of tissue hypoxia during cardio-pulmonary bypass [28]. Erythropoietin (EPO) is released to the plasma in the presence hypoxic injury to the kidney. A rise of this hormone was correlated with the onset and severity of anemia, suggesting that EPO could be a potential biomarker for the need for transfusion to avoid hypoxic injury to the kidney during cardio-pulmonary bypass [27]. The potential of EPO being a biomarker for the need of transfusion requires further exploration.

Recognizing the need for objective evidence-based markers for the need of transfusion, significant efforts have been directed at developing clinical trial-based guidance on the expected benefit of transfusion. The introduction of physiological, rather than [Hb] – based ones have been used as surrogates (e.g., heart rate ECG changes, mixed venous oxygen saturation, plasma lactate, etc.) [29]. A transfusion-attributed 10 g/L increase in [Hb] resulted in reduction of lactate clearance by >10% and increased central venous oxygen saturation by >5% in a third of the subjects [29]. Thus, there were putative physiologically meaningful benefits in *some but not all of the subjects*. There may be three conclusions from this study. First, that objective, physiological indicators can be applied to assess transfusion "efficacy" , and that a 10 g/L increment in a subject's [Hb] may offer a marginal benefit, and, lastly, that it confirms that not all individuals are alike in their responses and hypoxia tolerance.

This desired goal is hampered by the lack of objectively definable, universally applicable indicators to facilitate a *rational c*linical decision [12, 16].

The *expectation of benefit and of the efficacy of the transfusion* were important contributors to a degree of chaotic and individualistic approach to the use of transfusions, especially in surgical settings. Transfusion practices were variable, both among specialties and institutions, as well as within institutions. Many transfusions had been prescribed based on practitioners' personal values and expectations, as the true magnitude of the hazards of transfusion itself were not fully appreciated.

The Risks of Transfusion

Transfusion Reactions

Transfusion reactions as risk factors for adverse outcomes: these are adverse outcomes of a specified nature and had been well recognized.

(Chapter 6 of Part I of this book offers discussion of the nature, frequency, and clinical significance of *transfusion reactions* directly attributable to an incompatible transfusion.)

Transfusion reactions are identified *post facto* and their frequency, severity and their putative causes are monitored by national hemovigilance programs in most countries.

Transfusion reactions include [2, 3]:

- Incompatibility reactions to major or minor antigen mismatch, with or without hemolysis.
- Anaphylactic or allergic reactions [30].
- Accidental mismatch or preventable errors: wrong unit given to wrong patient.
- Transfusion Mediated Immune Modulation (TRIM) [31].
- Transfusion-Related Acute Lung Injury (TRALI) [32] and Transfusion-Associated Circulatory Overload (TACO) [33].
- Adverse reactions initiated by inflammatory mediators potentially derived from residual white cells remaining in transfused erythrocyte units.
- Febrile non-hemolytic transfusion reaction. Delayed serologic reaction.
- Post-transfusion purpura.
- Transfusion-Associated Graft vs Host reaction (T-A GVH) most likely affecting immunocompromised patients [34].

Fatal transfusion-related events occurring in the USA and reported to the FDA in the 5 years between 2012 and 2016 totaled sixty-five, of which one-half were hemolytic transfusion reactions. Despite the relatively low incidence of fatal transfusion reactions that should theoretically be preventable, these do happen and are a cause for concern [35]. The prevalence per 100,000 units transfused is reported yearly. This reporting is a great benefit in decisions of the statistical probabilities of assessing risk tolerance by both the prescriber and the patient.

Transfusion Reactions, TRALI, TRIM and T-A GVH) are rare but serious complications of transfusions.

In the surgical setting the immune suppression due to transfusion may be aggravated by immune suppression due tissue injury. In such cases the compelling argument favoring a transfusion are the consequences of the blood loss. Immune modulation is a well-known contributing risk factor for nosocomial infections in postoperative patients. Amelioration of the immune suppression may be a consideration for possible *avoidance* of transfusion, if feasible. Thus, the balancing of expected benefits and known and anticipatable risks is the *sine qua non* of a transfusion decision.

Residual leukocytes in erythrocyte units are thought to be a contributing risk factor to the pathogenesis of TRIM. Hence, increasing attention is directed at producing *leukoreduced* erythrocyte units. Comparison of transfusions of leukoreduced and non-leukoreduced units has shown true superiority of the former [36–39]. The ongoing universal implementation of leukoreduction and introduction of other specialized erythrocyte units (e.g., CMV-free units) became available and ameliorate these risk factors.

Transfusion Transmitted Infectious (TTI) Pathogens

In addition to the risk of transfusion reactions, another category of risks is the transmission of infectious pathogens present in donors, since blood cannot be sterilized [40]. The first infectious disease recognized to be transmissible by transfusion was syphilis and the Serologic Test for Syphilis (STS) was introduced in blood testing in 1935. The actual usefulness of this test is questionable, but it has remained in use. As refrigeration kills the *T. pallidum,* the clinical risk of syphilis has been overtaken by the more prevalent hepatitis viruses, transmissible by both blood and blood products, starting in 1965, which are not affected by refrigeration [1, 41].

The salient events in this regard in the USA were [1]:

- Hepatitis B surface antigen (HBsAg) discovered in 1965.
- Testing of blood donors for Hepatitis B surface antigen introduced in 1972.
- Transfusion-Associated Acquired Immunodeficiency Syndrome recognized in 1982.
- Donors deemed at high-risk behaviors were excluded in 1983.
- Human Immunodeficiency Virus (HIV) identified in 1984.
- HIV antibody testing introduced in 1985.
- Surrogate testing for hepatitis, (liver enzyme alanine transaminase ALT), hepatitis B antibody testing introduced in 1987.
- HTLV antibody testing introduced and hepatitis C virus identified in 1989.
- Hepatitis C testing introduced in 1990.[5]
- HIV 2 testing introduced in 1992.
- Nucleic acid testing and increasing numbers of rigorous virus testing introduced in the years following [1].

By the 1970s, the risks to the blood supply of potentially infectious *paid* donors were recognized, just as the demand for transfusions was increasing with the soaring number of coronary artery bypass graft (CABG) operations, starting in 1960. Many countries have made the shift away from paid to volunteer, unpaid, donors to protect the safety of their blood supply.

The HIV/AIDS Catastrophe

The arrival of the Human Immunodeficiency Virus (HIV) and its presence in the blood supply became the *third milestone* event.

The safety of the blood supply and of the erythrocyte and blood products became questioned with a panicked response by the population in most countries. People were unwilling to accept transfusions and untrue rumors circulating caused donations to plummet.

The tragic toll of potentially preventable illness and death became the stimulus for many countries to undertake rigorous and wide-ranging examination of the causes and the failures of national policy and response to the tragedy.

The Response to the Aids Crisis in Blood

The tragic toll exacted by the HIV and hepatitis viruses in the blood supply focused a searchlight on Transfusion Transmissible Infections (TTI) [41, 42], as a transfusion risk, distinct from transfusion reactions.

In the USA in the early 1980's 10,000 hemophiliacs and 12,000 other patients were infected by the HIV virus by blood and blood products and about 300,000 additional persons were infected with the HCV virus. To quote [43]:"The lessons from these tragedies compel greater vigilance and higher regulatory standards to protect the Nation's blood supply from emerging infectious agents and blood borne pathogens" [43–45]. Several policy recommendations were made to establish sub-Cabinet level Committees and Agencies to be responsible for protecting the safety of the blood supply, and these were to be established by statute. The introduction of new safety measures and policies to safeguard the blood supply was soon justified by the challenge of the emergence of a novel infectious agent, the Zika virus [46]. All donors, as of 2018, are screened by a high-performance Nucleic Acid Amplification Test (NAT) test. This newly emerged threat emphasized the importance of vigilance and horizon scanning to prevent the recurrence of a new HIV-like crises [47].

The introduction of new technologies for rigorous screening of blood, e.g., NAT testing, also introduced additional costs to the provision of blood for transfusion, to maximize blood safety.

NAT testing was first introduced as a sensitive and specific identifier of viral RNA or DNA to blood screening of donated units in the "window period" before infection could be detected by serological tests. They can be performed either on a single sample, or as multipacks combining a multiple of samples. NAT testing was first introduced in Germany in 1997, followed by the Netherlands in 2000. Approximately 33 countries use NAT testing on their blood collections.

NAT tests performed on multiple combined samples have the advantage of having the lower cost of fewer tests than tests performed on individual samples from all donations. Their disadvantage is that once a multipack is identified as positive, all units in the multipack sample need to be quarantined until further tests are completed to identify the one positive unit and the rest can be released [48]. The alternative to testing multipacks is testing of single units; this will

[5]The 2020 Nobel prize in Physiology or Medicine was awarded to H.J Alter, H Houghton and C.M. Rice for the discovery of the Hepatitis C Virus: https://www.nobelprize.org/prizes/medicine/2020/press-release/

increase the test's sensitivity, but also multiplies the total costs, while avoiding delays in releasing non-reactive units.

Cost-effectiveness in pharmacoeconomic analysis uses the metric of incremental cost of Quality Adjusted Life Years (QALY) achieved. Pharmaco-economic analysis was performed in several countries following the implementation of NAT testing. In the United States, [49] the study found that, using minipool samples, NAT testing would avoid an estimated 37, 128, and 8 cases of HBV, HCV, and HIV, respectively, and would add 53 additional years of life, and 102 additional QALY, compared with single samples tested at a net cost of $ 154 million. For relative scale, note that approximately eight million units are transfused annually in the USA. The incremental cost ratio estimated was $1.5 million per QALY gained. The authors concluded that the cost-effectiveness of adding NAT screening in the US blood system would be outside the typical range of most health care interventions, but not for established blood safety measures.

Following the introduction of NAT testing in Germany in 1997, the German Red Cross reviewed its experience with NAT testing the German blood supply [50]. In the eight-year period (1997-2005) 30.5 million donations (representing about 80% of the total blood collected) were tested. A total 27 HCV, seven HIV-1, and 43 HBV positives had been detected that would have been missed by serological methods only. Thus, NAT testing applied in the "window period" found that the residual risk per unit transfused was estimated at 1 in 10.88 million units for HCV, 1 in 4.3 million units for HIV-1, and 1 in 360,000 units for HBV. The authors concluded that the risk avoided by the addition of NAT testing was "very low", at a substantial cost.

A third study conducted in Zimbabwe shows how extreme inequality between high- and low-income countries affects these policy decisions [51]. The estimated prevention of infections by the addition of NAT testing would be 25, six, and nine HBV, HCV, and HIV infections, respectively. The incremental cost was estimated at US $ 17,774 for each QALY achieved. This is three times the gross per-capita income in Zimbabwe and fails the test of a reasonable cost.

Thus, the mandates to maximize the safety of the blood supply in high-income countries come at high cost that is felt to be within their national priorities in maintaining the safety of blood. It is clearly an impossibility in countries with low incomes and failed economies.

Canada's blood system was severely impacted by the HIV crisis. And the failure of a timely response to introduce testing blood collected for the hepatitis virus, as a surrogate, before the identification of the HIV virus. The panic had been aggravated and the tragedy amplified. Criminal charges had been filed against several individuals deemed to be responsible for the delays in recognition of the threat and failing to act in a timely manner. At trial, those charged were not convicted. Those responsible, however, were confronted by many of the victims, and the participation of victims in the review of the events provided those affected an opportunity to express their grief.

A wide-ranging and clear-eyed examination of all the factors was undertaken by a Royal Commission under Mr. Justice Horace Krever over 3 years and costing CDN $ 17 million [52]. The three volumes and appendix take an enormously expansive look at all aspects of the provision of all blood products and components and the means available for reducing contamination. The policy recommendations were far reaching. Before the crisis, the Canadian Red Cross managed all aspects of donor recruitment, donations and processing of blood and components, except for apheresis collection and processing blood products.

The Commission recommended a complete reorganization of all aspects of Canada's blood system and all its recommendations were implemented by statute. The Canadian Red Cross lost all participation in managing the blood system. Blood, blood products and components were to be treated not as commodities but as taxpayer-funded public goods. A completely new organization, Canadian Blood Services (CBS), was set up on a nation-wide scale, to become the overall manager of blood collection from volunteer donors only, all aspects of processing and supply and to include under its aegis organ transplants and stem cells, as well. Cord blood collection remained in private hands. No blood and blood components are imported to Canada, and blood products are imported only after heat treatment. The CBS encompassed all Canadian provinces and territories, except for Quebec where a similar organization, Hema-Quebec, fulfills a similar mandate. CBS' s global budget is funded from provincial and federal contributions on an annual basis and is overseen by a council of all Health Ministers. Hema-Quebec receives its funding from the Province and the federal government. CBS provides hospital blood banks and other blood users all blood and components free of charge and recipients are not charged for any services. Blood products for hemophiliacs are provided free by the provincial health insurance agencies.

CBS screens all blood collected with NAT testing for HIV-1 and 2, HBV and HCV, as well as for West Nile Virus during the summer season and for Chagas' disease (*T. cruzi*) in travelers.

Volume 3 of the Krever Report provides an exhaustive review of international events and national blood systems, including those of the USA, and comparisons made between systems.

Ten years following the Report, an appraisal concluded that the reform of the Canadian blood system was successful. The public has been kept safe from transfusion transmissible infectious threats by rigorous screening and deferral of potential high-risk donors, by an all-volunteer loyal donor base [53].

Two non-fiction books by Canadian journalists tell the story of those affected in Canada [54, 55].

The World Health Organization (WHO) publishes periodic reports on blood safety and availability in most countries [56].

Thus, the milestone event of the HIV crisis focused attention on transfusion transmissible infections (TTI). This also meant that TTI's came to be recognized as the second category of serious risk of transfusions, in addition to transfusion reactions. In many countries national policies were introduced mandating maximal efforts to safeguard the scarce and precious resource. The public policy to restore the public's trust in the safety of blood by a costly effort has been successful in many countries.

As if to reinforce that the emergence of novel and rare infectious disease threats requires continued vigilance and rapid response, the Zika virus arose from Micronesia and was brought to Brazil by Olympic athletes from French Polynesia. Sporadically reported from Africa and Asia before, this mosquito-borne virus attacked an immunologically naïve population in Brazil and caused the birth of thousands of microcephalic infants. The arrival of the virus in 2015 caused an international public health emergency [47]. Infected adults have viremia, but 80 percent are asymptomatic, spreading the virus widely [57]. Potential viremic blood donors without symptoms would threaten the blood supply if sensitive testing were not introduced promptly. While a NAT based test became available in Brazil, not all blood centers had been required to introduce it universally. Apparently, a few cases of transfusion transmitted infections have been reported, although the overwhelming majority of infections did not enter the blood supply. According to AABB[6] criteria, the virus should be classified as a high-risk infectious agent [46]. Whereas most infections cause no symptoms, the virus is also implicated in rare cases of Guillain-Barre syndrome. Hence, recipients of infected units are at risk of serious but rare complications. The Zika virus is another infectious agent that poses threats to the blood supply in endemic areas, posing challenges to blood collection [58].

In the USA FDA issued Guidance in August in 2016 recommending universal NAT testing for Zika in blood donors. By then, more than 4000 travel related Zika infections had been reported to the Centers for Disease Control and Prevention (CDC) [57].

As health care costs escalate in most countries, the distribution of scarce resources, including financial ones, become important considerations. Pharmaco-economic analysis is being applied to aid decision-making about resource allocation. Among these, the mandate to assure the attainment of best available safety of the blood supply is also constrained by the escalating costs associated with the introduction of newer tests mandated and more expensive technologies introduced. Economic considerations have been applied to blood processing and transfusion-associated costs [59]. As the effectiveness of transfusion has been often overestimated, whereas the risks have been underestimated; cost-effectiveness of transfusion as a frequent medical-surgical intervention needs to be examined [59].

[6]American Association of Blood Banks

Transfusion-Attributable Adverse Outcomes

The fourth Milestone event: It is being recognized that those receiving transfusions are at risk for adverse outcomes that occur more frequently than in those who had not been exposed to a transfusion. These adverse outcomes are recognized, based on presumptive evidence, as the *third category of risks affecting transfusion recipients, in addition to transfusion reactions and TTI's.*

Jehovah's Witness patients undergoing cardiac surgery are an instructive cohort to consider, when compared to patients undergoing similar procedures who also receive transfusion. Cardiac surgery patients are good examples, because they are at high risk of needing a transfusion, due to uncontrolled bleeding, anticoagulant use, and coagulation defects. A statistical tool, "propensity matching", enables the selection, from a large cohort, patients who are closely comparable to a smaller cohort when the two cohorts differ in a single attribute, namely, whether or not exposed to transfusion. The study from the Cleveland Clinic [14] reviewed retrospectively in a seven-year period 87, 775 consecutive cases undergoing Coronary Artery Bypass Grafting (CABG). Of this population, 56% (48, 986) received transfusion(s). Using propensity matching, the study selected 322 transfused patients who matched 322 Jehovah's Witness untransfused patients. The matching created two comparable cohorts of equal size, comprising of patients who were like each other with respect to many preoperative and operative characteristics, but differed with respect to transfusion exposure. During the 30-day postoperative period there were 14 deaths in the transfused and 10 deaths in the untransfused Witness patients (14/322; 4.3%, vs 10/322; 3.1%; Not significantly different). However, significantly more *adverse events* of myocardial infarction, respiratory failure and reoperations occurred in the transfused cohort. Indicative of the severity and frequency of adverse outcomes, longer ICU, and operative hospital lengths of stay (LOS) were also seen in the transfused patients. Long term survival of those followed up also favored the Witness patients.

A study deploying similar methodology also found differences in adverse outcomes experienced between transfused and untransfused patients as those in the Cleveland Clinic study above [60]. This study population comprised two cohorts of 857 matched pairs. More of the transfused patients experienced myocardial infarctions, respiratory and renal failure, reoperations and longer ICU and hospital LOS. These

comparative studies, while not definitive, do suggest that the exposure to transfusion may be a contributing risk factor to more frequent adverse outcomes. This introduces the concept that *transfusion avoidance* may be a desirable clinical goal, avoiding some of the excess risks that transfusion recipients may experience, leading to the fifth milestone.

Transfusion Avoidance and Blood Conservation

From the foregoing narrative it is evident that there are benefits and risks to be considered when a transfusion decision is made. Thus far, both have been considered in the abstract, without regard to the severity of the anemia of the patient, and its risks. From the consideration of risks of anemia above, it is evident that a [Hb] less than 50–60 g/L is a significant threat to survival. Even that threshold may be dependent of the patient's physiological reserves and resilience.

To summarize the intertwined risks and benefits of anemia and transfusion:

- SEVERE ANEMIA:
  - If untreated, is a threat to health and survival.
  - Is a risk factor for transfusion,
  - Is potentially improved by transfusion by avoiding anemia threats.
- TRANSFUSION:
  - Has inherent risks: transfusion reactions, transmitted infections, transfusion-attributable enhanced risk of adverse outcomes.
  - There is benefit in avoiding transfusion: avoid above risks to individual.
  - Benefits to community: Conserve scarce resources: blood, financial.

The predictive importance of preoperative anemia was evaluated in a cohort of 33,411 patients undergoing elective cardiac surgery [61]. Thirty-one percent (n = 10, 357) of these patients received transfusion(s) indicating how frequently transfusions are prescribed in these circumstances. The likelihood of transfusion was correlated with preoperative anemia. The adjusted mortality rate and a greater number of adverse outcomes was correlated with receipt of transfusion. This indicates that transfusion is an independent risk factor for additional adverse outcomes [62, 63].

In each case, an additional consideration of cost differences may also apply [64, 65] (see below).

Following the recognition of transfusion associated risks to the patients, the then (1997) available guidelines for the use of erythrocyte transfusions were reviewed [66]. The review found no expert consensus-based guideline or practice recommendation for objective guidance for erythrocyte transfusions.

In 2011 a paper appeared in the British Journal of Anaesthesia with the provocative title, "*What is really dangerous: anaemia or transfusion?"* [67] Shander and colleagues reviewed the physiological mechanisms available to protect from hypoxic tissue and organ injury and called for further research to characterize these risks to better enable rational transfusion decisions that minimize risks and maximize benefits.

The Search for an Objective Transfusion "Trigger"

The fifth milestone:

The Transfusion Requirements in Critical Care (TRICC) Study

The first randomized controlled clinical trial intended to find *objectively definable transfusion "triggers"* in ICU patients, appeared in the New England Journal of Medicine, February 11, 1999 [68]. The study was intended to find non-inferiority between two groups of critical care patients in 22 tertiary care and three community hospitals in Canada and the USA. The two groups were randomized to receive daily transfusions, to maintain their [Hb] either within the range of *70–90 g/L* in the so-called *restrictive* cohort, and within the range of *100–120 g/L* in the so-called *liberal* cohort.

Carefully selected inclusion/exclusion criteria and characterization of each subject's pre-randomization profile (using such as Multiple Organ Dysfunction Scores (MODS)) [69] were recorded, to allow clinical comparison of the two cohorts, as well as daily measures during the trial to compare outcomes between the two cohorts. The enrolled population was randomized one-to-one into either the restrictive or the liberal transfusion cohort (n = 418 and n = 420), respectively. Primary outcome measures were mortality at various time points.

The subjects were successfully maintained at their assigned [Hb] ranges (85 ± 7 and 107 ± 7 g/L, $p < 0.01$). Mortality rates in the ICU and at 30 days were lower in the restrictive than in the liberal groups. The mean number of transfusions received was significantly different between the groups; 2.6 ± 4.1, vs 5.6 ± 5.3 units per subject in the restrictive and liberal groups, respectively. The difference between the groups was also evident in the total number of transfusions *avoided*: 138 of the 418 subjects in the restrictive group (33%) entirely avoided transfusion, whereas all subjects in the liberal group received at least one transfusion. The transfusions avoided by the restrictive group subjects represented a 46% reduction in total number of transfusions. The clinical

severity scores at entry to the trial also predicted that those less severely ill subjects, who were <55 years of age, were able to tolerate relative anemia better, and were less likely to experience adverse outcomes.

The demonstration of non-inferiority of the two treatment strategies indicated that an objective transfusion "trigger" can be found for transfusion decisions in critical care patients. Moreover, that patients, with appropriately triggered transfusions in the range of [Hb] of 70–90 g/L, do not experience more severe outcomes than those receiving transfusions triggered in the [Hb] range of 100–120 g/L. In the former group of patients, the actual avoidance of transfusions did not increase their risk of anemia-related adverse outcomes. Thus, using an objective "diagnostic indicator" for the need of transfusion can contribute to blood conservation, without apparently sacrificing patient safety. And, finally, the study showed that it is not necessary to restore patients' [Hb] to the reference "normal" range of 140–150 g/L, and that the "breakpoint" of ≤50 g/L being predictive of severe risks, noted above, on the one hand, and the tolerable level ≥70 g/L on the other, shows a relatively narrow band for risk tolerance in intensive care.

Of course, the TRICC study may not be fully generalizable to all critical care patients. Those suffering from coronary artery disease may be especially vulnerable to the risks of anemia [15]. In fact, subgroup analysis of the TRICC study found [70] that in those subjects with severe cardiovascular disease, more prudent use of liberal thresholds may be beneficial.

The avoidance of unnecessary transfusions and of transfusion-related adverse outcomes may have salutary financial benefits, as well. A theoretical model published in 2007, estimated that of the then current 3.070 million units transfused annually in critical care patients in the USA, universal adoption of the restrictive transfusion threshold would be reduced to 1.778 million units, a reduction of 42%. The model also estimated an avoidance of 1624 severe transfusion-attributable adverse outcomes, a reduction of 69%. Using an average unit cost of US $634, the estimated annual cost saving would be US$ 821 million, or 42%. if all ICU transfusion decisions were based on the restrictive thresholds [71]. While these numbers are far out of date, their message is significant: substantial savings could be achieved by restricted use of transfusion, and patients could avoid a significant number of transfusion-attributable adverse outcomes, all the while conserving blood and financial resources.

The findings of the TRICC study have been confirmed in similar, large scale randomized trials in Europe. No significant difference in mortality rates were found between restrictive and liberal transfusion strategies, and significant blood sparing was demonstrated [72]. Meta-analyses of published trials comparing low and higher transfusion thresholds have also confirmed the general conclusions [73–79]. A more recent meta-analysis paying particular attention to patients with cardiovascular disease recommended a more cautious approach to this population [80]. Longer range (6 months) outcomes after discharge from hospital of anemic patients were assessed and found to be not different among recipients of transfusions triggered by the two strategies [81, 82].

A systematic review of available meta-analyses provided an overview of all reviews comparing mortality in restrictive and liberal [Hb]-based thresholds [83]. This review comprised 33 meta-analyses of variable quality. Among good and moderate quality analyses (total 16), found lower mortality among subjects assigned to the restrictive transfusion thresholds. Thus, a large and diverse set of subjects, from a diversity of institutions, who were transfused at restrictive thresholds, did not experience greater mortality than those transfused at liberal thresholds.

Guidelines recommend that transfusions be used sparingly in critical care units and to avoid excessive phlebotomies and to use alternatives to transfusion, such erythropoietin [84, 85]. Separate guidelines have been published on transfusion support for CABG patients, recommending more frequent use of preoperative autologous transfusion, blood salvage and the establishment of multidisciplinary approach to use interventions that avoid allogeneic transfusions [86].

A major review evaluated whether the two transfusion thresholds are associated with different risks of health-care associated infections [87]. The pooled risk of infection acquired in the health care setting was 10.6% in the restrictive and 12.7% in the liberal transfusion threshold cohorts. The relative risk (RR) for *all* infections was 0.92 ((95% confidence interval, CI, 0.82–1.04) was not significantly different between thresholds applied. The RR for *serious* infections was 0.84 (95% C.I 0.73–0.96) was significantly higher for the liberal transfusion thresholds applied. No difference was found between leukocyte-reduced and non-leukocyte reduced units transfused.

An attempt was made to review all available studies comparing restrictive and liberal transfusion thresholds for transfusion in surgical and critical care settings [88]. The review comprised 31 trials involving 12,587 participating subjects. The studies used either a [Hb] of 70 g/L for restrictive transfusion triggering, or [Hb] of 80–90 g/L threshold for liberal triggering. The cohorts comprising the two thresholds used were approximately evenly matched. Use of the restrictive threshold reduced the probability of receiving a transfusion by 43%, while it neither increased nor significantly decreased 30-day mortality, or any other adverse outcomes assessed when compared to the liberal transfusion cohort. The authors concluded that transfusions applied at the restrictive threshold reduced the risk of receiving a transfusion without significantly altering the subjects' other risks.

Restrictive vs Liberal Transfusion in Cardiac Surgery

Coronary Artery Bypass Grafting (CABG) surgery is the numerically largest subset of cardiac surgery and accounts for significant intra- and postoperative transfusions, cumulatively a large proportion of surgical transfusions overall.

A large prospective trial compared outcomes in 4860 subjects undergoing cardiac surgery in Canada, Australia, and New Zealand (TRICC III Study) [76]. The subjects were randomized one-to-one into, either *restrictive* or *liberal* transfusion arms. The transfusion thresholds were *[Hb] <75 g/L* in the former, and *[Hb] <95 g/L* in the latter. The two groups were comparable with respect to their preoperative demographic and clinical profiles and surgical procedures. Composite outcome measures were death, stroke, myocardial infarction, and new renal failure. All primary outcomes were comparable between the two arms. One thousand, one hundred and fifty-nine subjects (48%) avoided transfusion in the restrictive arm, as opposed to only 663 in the liberal arm. Thus, twice as many liberal-arm subjects than those in the restrictive-arm received *intraoperative* transfusions. Significantly more liberal-arm subjects were exposed to *postoperative* transfusions (52% vs 36%). In the total population of 4860 subjects, a total of 8987 erythrocyte units had been consumed, with a markedly uneven distribution: 3486 units in the restrictive arm and 5501 units in the liberal arm; over 2000 units had been saved in the restricted arm subjects, who had not suffered significantly worse outcomes. The only substantive difference observed was a longer aggregate ICU LOS time (9.7%) in the restrictive-arm subjects. The excess ICU costs in this cohort may be offset by the saving of 2000 unused transfusion units. Long range outcomes in anemic patients discharged from hospital were also found be similar [63, 82].

A systematic review and meta-analysis of 13 similar randomized controlled trials, including the TRICC study followed [89]. The review comprised 9092 patients undergoing cardiac surgery. The adjusted risk ratios for mortality, myocardial infarction, stroke, and arrhythmia were all similar between restricted and liberal transfusion treated subjects. Unlike in the TRICC III study above, both aggregated ICU and hospital LOS were similar. While all other observed risk ratios were similar, the risk of receiving an erythrocyte transfusion favored the subjects in the restrictive treated cohort [90].

In summary, numerous randomized controlled trials and meta-analyses support a restrictive approach to transfusions at thresholds of [Hb] 70–75 g/L, that permits either avoidance or minimizing transfusion exposure among cardiac and other surgical patients. Thus, rational, evidence-based avoidance of unnecessary erythrocyte transfusion minimizes transfusion-attributable risks, spares blood resources and does not expose patients to excessive anemia-related risks.

Systematic reviews have been published in cardiac surgery in children with congenital heart disease, [91] and in neurocritical adults [92].

Another systematic review and meta-analysis also found consistent similarities in outcomes between subjects treated with restrictive and liberal transfusion strategies [93]. One exception to this consistent trend of similar outcomes was two small trials (n = 154 subjects) with acute myocardial infarction in which the liberal transfusion strategy appeared more favorable [80]. Similar caution of favoring more liberal transfusion thresholds in patients with cardiovascular disease, undergoing non-cardiac surgery [80] is recommended.

There have been critics of such clinical trials conducted on intensive care subjects, because of their diversity [94–98].

The alternative to transfusions in surgical practice is *bloodless surgery*. This practice utilizes meticulous hemostasis and attention to coagulation, and offers the advantages of avoiding transfusions, as well as the adverse outcomes associated with it [99].

This provides the transition to the sixth and last milestone:

Patient Blood Management

The transfusion landscape convinced Shander and colleagues to propose an entirely new paradigm for the use of transfusion [100]. It proposed that instead of treating the *Hb concentration* of an anemic patient, the *patient* with anemia should be treated. Prudent use of "this lifesaving, costly, limited and dangerous resource", transfusion, was required [100].

Shander and colleagues posited that "the vast majority of transfusions in surgical patients can be attributed to low preoperative hemoglobin levels, excessive surgical blood loss and /or inappropriate transfusion practices" [99].

The multimodal Patient Blood Management Program (PBM) is conceived as resting on three pillars: [100–102].

- Optimizing hematopoiesis.
- Minimizing operative and other blood losses.
- Harnessing and optimizing physiological tolerance of anemia.

The rationale of a transfusion to treat anemia is based on the determinants of oxygen delivery, i.e., cardiac output, and oxygen content of the blood. The latter is determined by the [Hb]. Of these two, only the cardiovascular adjustments can be altered at will, whereas increasing hemoglobin concentration by erythropoiesis is slow. Thus, the first pillar demands *preoperative* attention to [Hb]. The first and third pillars above can be manipulated by the clinician's pharmacological

armamentarium. The second pillar demands meticulous operative technique of hemostasis and immediate correction of coagulation disorders. When the three pillars do not offer sufficient relief, recourse to transfusion is based on evidence-based assessment of the attendant risks, taking into consideration of anemia tolerance of the patient in question.

The proposal is based on the recognition that hidden anemia is common, especially in the elderly and many disadvantaged groups. When anemic patients require surgical or critical care treatment, they have often required transfusion. The benefits of the new program lie in minimizing risks, conserving scarce resources, as well as controlling some costs in anemic patients who subsequently require surgical or critical care.

The scientific basis for Patient Blood Management (PBM) was laid out in 2015 [103]. The concept is defined as: comprising …"measures to avoid transfusion such as anemia management without transfusion, cell salvage and the use of anti-fibrinolytic drugs, to reduce bleeding as well as restrictive transfusion" only if needed. "It ensures that patients receive the optimal treatment, and that avoidable, inappropriate use of blood and components is reduced." The concept has become widely implemented in Europe [104].

Thus, the prevention of anemia is now a desirable objective of medical care. It requires a high degree of cooperation by many disciplines to assure that all therapeutic modalities are brought to bear to assure the best outcomes for the patient. To promote the acceptance and implementation of PBM's principles and practices, a new institute was formed: *The Institute for Patient Blood Management and Bloodless Medicine* [99, 101].

Several publications have assessed the PBM program, and these have been subject to systematic review [105]. The review comprised a total of 235,779 surgical patients reported in 17 studies published between 2008 and 2017, comparing 100,866 patients *before* the implementation of PBM *(pre-PBM*) and 134,893 *after* PBM was implemented *(post-PBM*). In the post-PBM population transfusion rates were 39 percent less than in the pre-PBM cohort, a mean 0.43 fewer erythrocyte units per subject were used, and hospital LOS was shortened by 0.45 days per patient. The total number of in-hospital days were reduced by 40% after PBM introduction, as the total number of complications were reduced by 20% and mortality rate decreased by 11%. The participating institutions used their institutional transfusion thresholds, but the "before-and-after" comparisons favors the conclusion that PBM introduced at many institutions resulted in cost and blood savings, improved patient outcomes, without disadvantaging those patients hospitalized post-PBM. It is admittedly possible that other than PBM practices also contributed to the observed differences between pre-PBM and post-PBM patients' outcomes. For example, hospital practices may have changed during the nine-year interval, favoring earlier discharge to prevent nosocomial infections. Other factors may also have contributed. Nevertheless, in a large and diverse patient population from many institutions at least, PBM practices were an important contributing factor.

If we accept that in the data above, PBM played an important role, then the observed average changes in outcomes can be seen in a different light. The reduction of 0.43 erythrocyte units may seem trivial, but in the aggregated more than 135,000 patients, it represents a total of 58,000 units saved. If this argument were extended to the total 216,657 patients, the savings would have exceeded 93,000 units, somewhat less than 1% of the total annual use of erythrocyte units. Likewise, the reduced mean individual shortened hospital LOS of 0.45 days per patient, applied across the 100,886 pre-PBM patients would aggregate to a total of 45,398 hospital days saved. These findings show clear benefits that can be attributed to the PBM program, without substantial excess in adverse outcomes.

Lastly, the review also identified the surgical specialties in which the greatest benefits could be expected. Orthopedic patients experienced the greatest reductions in transfusion exposure (55%) and mortality (27%). Cardiac surgical patients experienced the greatest reductions in number of units transfused (0.87 units per patient) and in-hospital LOS (1.34 days per patient).

The possible cost containment afforded by appropriate management of surgical transfusions that also avoid risks contributed by inappropriate transfusions, has major implications for the management of scarce health care resources. Pre-empting inappropriate transfusions by the PBM program is clearly indicated [59]. Orthopedic surgery accounts for 45% of surgical patients exposed to transfusion(s), accounting for about 10% of all erythrocyte units transfused. Preoperative transfusions in anemic patients have not been shown to be beneficial, as postoperative complications are not reduced. The implementation of PBM has clearly shown to be efficacious and has been successful in the USA. Western Australia's government is a leader in promoting efficient blood utilization with good results [106]. PBM is also implemented in most European countries. A review of its status in individual countries is given in [59, 104].

A direct case of cost containment can be made for reducing transfusion exposure and its attendant risks. A retrospective analysis of hospitalized patients in Australia compared costs incurred between transfused and untransfused patients [64]. In a total of 89,996 acute care hospitalized patients' costs were analyzed and subjected to multiple regression to eliminate confounding variables. Four thousand eight hundred and five patients were transfused (5.3%). This latter cohort incurred a mean 83% greater costs than the mean in untransfused patients. The study's specific findings may be questioned, as the receipt of transfusion(s) may be a surrogate marker for greater acuity, but the statistical analysis

attempted to account for this. The total transfusion-associated excess cost was equivalent to US $ 72 million, or about $ 15,000 per patient, far exceeding the direct cost of the transfusion(s) *per se*. Direct hospital costs of allogeneic, autologous, and perioperative transfusions were analyzed in Sweden [65]. The average direct cost of a two-unit transfusion was equivalent to approximately US $ 678.

The benefits of PBM have been recognized in numerous publications in the past 5 years. It is a fundamental shift away from a product-centered to a patient-centered approach to transfusion [99, 100, 102, 107–111]. International conferences in 2017 [112] and 2019 [113] have published guidelines for the implementation and practice of PBM.

The PBM program is consistent with the principles of sound risk management [114]. This publication reviewed the direct and indirect hazards associated with transfusions and provided medico-legal considerations in clinical risk management. It posited that PBM should be the state of the current art to avoid not only adverse outcomes for patients, but also to minimize the risk of litigation for practitioners and institutions. The authors noted that:

- Blood transfusion is now clearly known to have hazards that are avoidable, and PBM is the program currently most capable of minimizing those hazards.
- It is recognized that PBM is now the state of the art of surgical transfusion practice.
- Failure to follow current state of the art practice regarding transfusion may have deleterious clinical consequences that may also expose practitioners and institutions to enhanced litigation risk.

It is important to note that **transfusion avoidance is not withholding of necessary medical treatment.** Transfusion is still necessary in many circumstances, including continuing uncontrolled bleeding and when fluid resuscitation results in critically low [Hb], in cases of chronic severe anemia of bone marrow failure or chemotherapy, in the prevention of strokes in children with sickle cell disease and in hemoglobinopathies. But avoidance is an **ethically justifiable medical treatment decision when the risks and consequences of transfusion(s) outweigh a low level of risk of death.** The clinical trials showing non-inferiority of mortality outcomes in subjects having low [Hb] indicates that the risk of death due to avoiding transfusion is acceptably low at [Hb] greater than 70–80 g/L, except in those cases of severe coronary artery disease. A study in 2012 found that patients who had been transfused and with discharge [HB] of 100 g/L, or even 90 g/L "had received excessive transfusion" [115, 116]. The review of the risks of anemia and transfusion by Shander and colleagues [67, 78, 100, 117] has led them to the conclusion that in specific circumstances transfusion avoidance is medically and morally justified. The benefits also include blood resource conservation, but that reason *alone* is not morally justifiable to avoid transfusion. The question of whether a liberal transfusion threshold in elderly, non-cardiac surgical patients is capable of avoiding ischemic events, will be more definitively answered when the LIBERAL TRIAL results become available [118].

Summary and Conclusions

The discovery of major blood group antigens made it possible to choose donor blood for individual recipients that minimized the likelihood of severe transfusion reactions. In the early decades of the twentieth century, blood transfusion, like most other medical interventions, was used in the expectation of benefits to the recipient since anemia and bleeding were clearly viewed as threats to survival. As transfusion reactions became better understood, the consideration of expected benefit became tempered with consideration of the attendant risks.

The six milestones in the discussion above were the signal achievements of making transfusion of erythrocytes safer and more effective. These milestones stand out in the development of increasing safety of transfusions, as the three categories of transfusion risks – reactions, transmitted pathogens, and transfusion attributable adverse outcomes – were identified. Managing these risks became part of the evidence-guided use of erythrocyte transfusions. Maintaining the safety of blood resources after the HIV/AIDS epidemic became national priorities and large resource allocations were justified. Recognition of transfusion associated risks mandated that the prescribing of transfusions be subject to consideration of risks and benefits. Randomized controlled clinical trials defined objective criteria for [Hb] based "quantitative" thresholds and have been evaluated and found to offer relative safety from excessive adverse outcomes at [Hb] less than the "normal" levels. This facilitates safe, complete, or maximal possible, avoidance of transfusion exposure. The most recent development of the new paradigm of Patient Blood Management (PBM) program incentivizes optimal patient outcomes and the management of transfusions in populations that had previously been at high risk of surgical or critical care transfusion because they are chronically anemic. This large vulnerable population can be safely managed using the three pillars of PBM, namely hematopoietic management, minimizing blood loss and optimizing physiological adaptations to the presence of anemia. The immediate past decade has seen increasing acceptance of the practices embodied in PBM, by repeated demonstrations of its effectiveness in minimizing adverse outcomes, reducing transfusion exposures, saving scarce blood resources, and controlling costs. The principles of sound risk management

of all medical risks, including legal ones, suggests that these principles be accepted as the state of the current art.

Key Points

- The transfusion of erythrocytes remains the standard of care for correction of critical severity of anemia for the prevention of hypoxic end-organ injury.
- Since the introduction of blood transfusion, the procedure has not been subjected to critical evaluation of its efficacy, while its safety has been subject to increasing scrutiny during the past century.
- Three categories of safety risks have been identified: those of transfusion reaction directly attributable to a transfusion, those attributable to the transmission of infectious pathogens present in the transfusion, and those indirectly attributable and more frequently occurring adverse outcomes following transfusion exposures.
- The decision of whether a transfusion of banked erythrocytes is clinically advisable has become a matter of risk management whereby the benefit of avoiding predictable risks of the exposure is balanced against the risk of hypoxic end-organ injury.
- Erythrocyte transfusion remains the key treatment for continuing uncontrolled blood loss, erythropoietic failure of various causes, sickle cell disease and hemoglobinopathies. Surgical and critical care transfusions are guided by the principal tenets of Patient Blood Management which is an ethically justifiable avoidance of exposure to a transfusion with low level risks and consequences, while maintaining physiological means of improving oxygen supply.

Acknowledgement The author declares no interest in any commercial enterprise in this field.

References

1. Leonard K, Davey R. Chapter 12: principles of transfusion medicine. In: Lake L, Moore R, editors. Blood: hemostasis, transfusion, and alternatives in the perioperative period. 1st ed. New York: Raven Press; 1995. p. 229–75.
2. Popovsky M, editor. Transfusion reactions. 1st ed. Bethesda: AABB Press; 1996.
3. Popovsky M, editor. Transfusion reactions. 2nd ed. Bethesda: AABB Press; 2001.
4. Kendrick D. Blood program in World War II. US Army Medical Department. Washington, DC: Surgeon General, US Army.
5. Boulton F. Blood transfusion and the World Wars. Med Confl Surviv. 2015;31(1):57–68.
6. Goldman A, Schmalstieg F. Karl Otto Landsteiner; Physician-biochemist-immunologist. J Med Biogr. 2019;27(2):67–75.
7. Kyle R, Shampo M. Karl Landsteiner – discoverer of the major human blood groups. Mayo Clinic Proc. 2001;76:860.
8. Zetterstrom R. The Nobel Prize for the discovery of human blood groups: start of the prevention of haemolytic disease of the newborn. Acta Pediatr. 2007;96:1702–9.
9. Kantha S. The blood revolution initiated by the famous footnote of Karl Landsteiner's 1900 paper. Ceylon Med J. 1995;40(3):123–5.
10. Bodner W, McKie R. The book of man: the quest to discoverour genetic heritage. Toronto: Viking Penguin; 1994. p. 259.
11. Levine P, Stetson R. An unusual case of intra-group agglutination. J Am Med Assoc. 1939;113(2):126–7.
12. Hare G, Tsui A, Ozawa S, Shander A. Anaemia: can we define haemoglobin thresholds for impaired oxygen homeostasis and suggest new strategies for treatment? Best Pract Res Clin Anaesthesiol. 2013;27:85–98.
13. LaPar D, Hawkins R, McMurry T, Isbell JM, et al. Preoperative anemia versus blood transfusion: which is the culprit for worse outcomes in cardiac surgery? J Thorac Cardiovasc Surg. 2018;41:66–74.
14. Pattakos G, Koch C, Koch BM, Batizy L, et al. Outcome of patients who refuse transfusion after cardiac surgery; a natural experiment with severe blood conservation. Arch Intern Med. 2012;172(15):1152–60.
15. Guinn N, Cooter M, Villalpando C, Weiskopf R. Severe anemia associated with increased risk of death and myocardial ischemia in patients declining blood transfusion. Transfusion. 2018;58(10):2290–6.
16. Hare G, Tsui A, McLaren A, Ragoonanan T, Yu J, Mazer C. Anemia and cerebral outcomes: many questions, fewer answers. Anesth Analg. 2008;107(4):1356–70.
17. Weiskopf R, Kramer J, Viele M, et al. Acute severe isovolemic anemia impairs cognitive function in memory in humans. Anesthesiology. 2000;92:1646–52.
18. Weiskopf R, Feiner J, Hopf H, Viele M. Oxygen reverses deficits of cognitive function and memory and increased heart rate induced by acute severe isovolemic anemia. Anesthesiology. 2002;96(4):871–7.
19. English S, McIntyre L. Is hemoglobin good for cerebral oxygenation and clinical outcomes in acute brain injury? Curr Opin Crit Care. 2018;24(2):91–6.
20. Tsui A, Marsden P, Mazer C, Sled J, Lee K, Henkelman R, et al. Differential HIF and NOS responses to acute anemia: defining organ-specific hemoglobin thresholds for tissue hypoxia. Am J Physiol Integr Comp Physiol. 2014;307:R13–25.
21. Bahrstein G, Arbell D, Yedgar S. Hemodynamic functionality of transfused red blood cells in the microcirculation of blood recipients. Front Physiol. 2018;9:9–41.
22. Kim-Shapiro D, Lee J, Gladwin M. Storage lesion. Role of red cell breakdown. Transfusion. 2011;51(4):844–51.
23. Bahrstein G, Pries A, Goldschmidt N, Zukerman A, Orbach A, et al. Deformability of transfused red blood cells is a potent determinant of transfusion-induced change in the recipient's blood flow. Microcirculation. 2016;23(7):479–86.
24. Barshtein G, Manny N, Yedgar S. Circulatory risk in the transfusion of red blood cells with impaired flow properties induced by storage. Transfus Med Rev. 2011;25(1):24–35.
25. Kaul D, Kopshkaryev A, Atrman G, Bahrstein G, Yedgar S. Additive effect of red blood cell rigidity and adherence to endothelial cells inducing vascular resistance. Am J Physiol Heart Circ Physiol. 2008;295(4):H1788–93.
26. Tinmouth A, Fergusson D, Yee I, Hebert P, ABLE Investigators; Canadian Critical Care Trials Group. Clinical consequences of red cell storage in the critically ill. Transfusion. 2006;46(11):2014–24.
27. Hare G, Han K, Leschchyshyn Y, et al. Potential biomarkers of tissue hypoxia during acute hemodilutional anemia in cardiac surgery: a prospective study to assess tissue hypoxia as a mechanism of organ injury. Can J Anesth. 2018;65:901–13.
28. Stafford-Smith M, Grocott H. Renal medullary hypoxia during experimental cardiopulmonary bypass: a pilot study. Perfusion. 2005;20:53–8.

29. Fisher D, Mutlag H, Landau S, Mueller M. Effect of red blood cell transfusion on physiologic transfusion triggers in intensive care patients. Anesth Intensivemed. 2018;59:1–7.
30. Madden K, Raval J. Chapter 4.: Allergic transfusion reactions. In: Immunologic concepts in transfusion medicine [Internet]. Elsevier; 2019. p. 45–56.
31. Maitta R. Transfusion-related immunomodulation. In: Immunologic concepts in transfusion medicine [Internet]. Elsevier Pub; 2019. p. 81–90.
32. Harrold I, George MR. Transfusion-related acute lung injury. In: Immunologic concepts in transfusion medicine [Internet]. Elsevier Pub; 2019. p. 97–102.
33. Semple J, Rebetz J, Kapur R. Transfusion-associated circulatory overload and transfusion-related acute lung injury. Blood. 2019;133:1840–53.
34. Ryder A, Zheng Y. Graft-versus-host disease and transfusion-associated graft-versus-host disease. In: Immunologic concepts in transfusion medicine [Internet]. 1st ed. Elsevier Pub; 2019. p. 197–202.
35. Vamvakas E, Blajchman M. Transfusion-related mortality: the ongoing risks of allogeneic blood transfusion and the available strategies for their prevention. Blood. 2009;113:3406–17.
36. Hebert P, Fergusson D, Blajchman M, Wells G, et al. Clinical outcomes following institution of the Canadian Universal Leukoreduction Program for red cell transfusion. JAMA. 2003;289:1941–9.
37. Fung M, Rao N, Rice J, Ridenour M. Leukoreduction in the setting of open heart surgery: a prospective cohort-controlled study. Transfusion. 2004;44:30–5.
38. Simancas-Racinas D, Osorio D, et al. Leukoreduction for the prevention of adverse reactions from allogeneic blood transfusion. Cochrane Database Syst Rev. 2015;12:CD0097545.
39. Abdelsattar Z, Hendren S, Wong S, Campbell D Jr, Henke P. Variation in transfusion practices and the effect on outcomes after noncardiac surgery. Ann Surg. 2015;262(1):1–6.
40. Hong H, Pessin M, Barady E. Changing landscaping in transfusion-transmitted infections. In: Immunologic concepts in transfusion medicine [Internet]. Elesevier Pub.; 2019. p. 55–65.
41. Dzik WH. The changing Landscape of transfusion medicine: 2004–2015. Transf Med. 15(1):69–82.
42. Hong H, Pessin M, Babady E. Changing landscape in transfusion-transmitted infections. 2020. In: Immunologic concepts in transfuion medicine [Internet].
43. Oversight CoGRa. Protecting the Nation's Blood Supply from Infectious Agents: the need for new standards to meet new threats. Washington, DC1996 [Report].
44. Medicine Io. New measures needed to protect Nation's blood supply from future threats posed by infectious diseases. Washington, DC1995 [Report].
45. Global status report on blood safety and availability 2016. Geneva: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO. [Internet]. 2016.
46. Musso D, Stramer S, Busch M. Zika virus: a new challenge for blood transfusion. Lancet. 2016;387(10032):1993–4.
47. Bloch E, Ness P, Tobian A, Sugarman J. Revisiting blood safety practices given emerging data about Zika virus. NEJM. 2018;378:1637–841.
48. Hans R, Marvaha N. Nucleic acid testing – benefits and constraints. Asian J Transfus Sci. 2014;8(1):2–3.
49. Marshall D, Kleinman S, Wong J, Aubuchon J, Grima D, Kulin N, et al. Cost-effectiveness of nucleic acid test screening of volunteer blood donations for hepatitis B, hepatitis C and human immunodeficiency virus in the United States. Vox Sang. 2004;86(1):28–40.
50. Hourfar M, Jork C, Schottstedt V, Weber-Schehl M, et al. Experience of German Red Cross blood donor services with nucleic acid testing: results of screening more than 30 million blood donations for human immunodeficiency virus-1, hepatitis c virus, and hepatitis B virus. Transfusion. 2008;48(8):1558–66.
51. Mafirakureva N, Mapako T, Khoza S, Emmanuel J, et al. Cost effectiveness of adding nucleic acid testing to hepatitis B, hepatitis C, and human immunodeficiency virus screening of blood donations in Zimbabwe. Transfusion. 2016;56(12):3101–11.
52. Krever H. Commission of enquiry on the blood system in Canada – Krever report. Ottawa: Canada; 1996. [cited 2020 Dec 01, 2020]. Available from: https://publications.msss.gouv.qc.ca/msss/en/document-000416/.
53. Kumanan W. The Krever Commission ten years later. Canad Med Assoc J. 2007;177:1387–9.
54. Parsons V. Bad blood; the tragedy of the Canadian tainted blood scandal. 1st ed. Toronto: Lester Publishing Ltd; 1995.
55. Picard A. The gift of death; confronting Canada's tainted-blood tragedy. Toronto: HarperCollins Publishers; 1995. 320 p.
56. WHO. Global report on blood safety and availability. 2020.
57. Goodnough L, Marques M. Zika virus and Patient Blood Management. Anesth Analg. 2017;124(1):282–9.
58. Musso D, Busch M. Zika virus: a new challenge for blood transfusion. Lancet. 2016;387(10032):1993–4.
59. Hofmann A, Ozawa S, Farugia A, Farmer S, Shander A. Economic considerations on transfusion and patient blood management. Best Pract Res Clin Anesthesiol. 2013;27(1):59–68.
60. Shaw R, Johnson C, Ferrari G, Zapolanski A, et al. Balancing the benefits and risks of blood transfusion in patients undergoing cardiac surgery: a propensity-matched analysis. Interact Cardiovasc Thorac Surg. 2013;17(1):96–102.
61. Blaudszun G, Butchart A, Klein A. Blood conservation in cardiac surgery. Transfus Med. 2018;28(2):168–80.
62. Rawn J. The silent risks of blood transfusion. Curr Opin Anaesthesiol. 2008;21(5):664–8.
63. Roubinian N, Escobar G, Liu V, et al. Trends in red blood cell transfusion and 30-day mortality among hospitalized patients. Transfusion. 2014;54(10 Pt. 2):2678–86.
64. Trentino K, Farmer S, Swain S, Burrows S, et al. Increased hospital costs associated with red blood cell transfusion. Transfusion. 2015;55(5):1082–9.
65. Glenngard A, Persson U, Soderman C. Costs associated with blood transfusions in Sweden: the societal cost of autologous, allogeneic and perioperative RBC transfusion. Transfus Med. 2005;15(4):295–306.
66. Calder L, Hebert P, Carter A, Graham I. Review of published recommendations and guidelines for the transfusion of allogeneic red blood cells and plasma. Can Med Assoc J. 1997;156(11 Suppl):51–8.
67. Shander A, Javidroozi M, Ozawa S, Hare G. What is really dangerous: anaemia or transfusion? Brit J Anaesth. 2011;107(S1):i41–59.
68. Hebert P, Wells G, Blajchman M, Marshall J, et al. A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group. N Engl J Med. 1999;340(6):409–17.
69. Marshall J, Sibbald W, Cook D, Roy P. The multiple organ dysfunction score (MOD) score: a reliable descriptor of a complex clinical outcome. Crit Care Med. 1992;20(Suppl).:S80 abstract.
70. Hebert P, Martin Y, Baljchman M, Wells G, et al. Is a low transfusion trigger safe in critically ill patients with cardiovascular diseases? Crit Care Med. 2001;29(2):227–34.
71. Zilverberg M, Schorr M. Effect of restrictive transfusion strategy on transfusion attributable severe acute complications and costs in the US ICUs; a model. BMC Health Serv Res. 2007;7(7):138–54.

72. Lelubre C, Vincent J, Taccone F. Red blood cell transfusion strategies in critically ill patients: lessons from recent randomized clinical trials. Minerva Anesthesiol. 2016;82(9):1010–6.
73. Holst L, Petersen M, Haase N, Perner A, et al. Restrictive versus liberal transfusion strategy for red blood cell transfusion: systematic review of randomized trials with meta-analysis and trial sequential analysis, meta-analysis. BMJ. 2015;350:h1354.
74. Spahn D, Spahn G, Stein P. Evidence base for restrictive transfusion triggers in high-risk patients. Transf Med Hemother. 2015;42:110–4.
75. Simon G, Carswell A, Thom O, Fung Y. Outcomes of restrictive versus liberal transfusion strategies in older adults from nine randomised controlled trials: a systematic review and meta-analysis. Lancet Haematol. 2017;4(10):e465–e74.
76. Mazer C, Whitlock R, Fergusson D, et al. Restrictive or liberal red-cell transfusion for cardiac surgery. N Engl J Med. 2017;377(22):2133–44.
77. Shehata N, Whitlock R, Fergusson D, Thorpe K, et al. Transfusion Requirements in Cardiac Surgery III (TRICS III): study design of a randomized controlled trial. J Cardiothorac Vasc Anesthesia. 2018;32(1):121–9.
78. Shander A, Javidrurzi M, Naqvi S, Aregbeyen O, et al. An update on mortality and morbidity in patients with very low postoperative hemoglobin levels who decline blood transfusion. Transfusion. 2014;54(10pt2):2688–95.
79. Patel N, Murphy G. Evidence-based red blood cell transfusion practices in cardiac surgery. Transfus Med Rev. 2017;31(4):230–5.
80. Carson J, Stanworth S, Alexander J, Roubinian N, et al. Clinical trials evaluating red blood cell transfusion thresholds: an updated systematic review and with additional focus on patients with cardiovascular disease. Am Heart J. 2018;200:96–101.
81. Mazer C, Whitlock R, Fergusson D, et al. Six-month outcomes after restrictive or liberal transfusion for cardiac surgery. NEJM. 2018;379(13):1224–33.
82. Roubinian N, Murphy E, Mark D, et al. Long-term outcomes among patients discharged from hospital with moderate anemia: a retrospective cohort study. Ann Intern Med. 2019;170(2):81–9.
83. Trentino K, Farmer S, Leahy M, Sanfilippo F, et al. Systematic reviews and meta-analyses comparing mortality in restrictive and liberal haemoglobin thresholds for red cell transfusion: an overview of systematic reviews. BMC Med. 2020;18:154.
84. Lasocki S, Pene F, Ait-Oufella H, Aubron C, et al. Management and prevention of anemia (acute bleeding excluded) in adult critical care patients. Ann Intensive Care. 2020;10:97.
85. Management. ASoTFoPB. An updated report. Anesthesiology. 2015;122(2):241–75.
86. Goodnough T, Johnston M, Ramsey G, Sayers M, et al. Guidelines for transfusion support in patients undergoing coronary artery bypass grafting. Ann Thorac Surg. 1990;50(4):675–83.
87. Rohde J, Dimcheff D, Blumberg N, Saint S, et al. Health care-associated infection after red blood cell transfusion: a systematic review and meta-analysis. JAMA. 2014;311(13):1317–26.
88. Carson J, Stanworth S, Roubinian N, Fergusson D, et al. Transfusion thresholds and other strategies for guiding allogeneic red blood cell transfusion. Cochrane Database Syst Rev. 2016:CD002042.
89. Shehata N, Mistry N, da Costa B, et al. Restrictive compared with liberal red cell transfusion strategies in cardiac surgery: a meta-analysis. Eur Heart J. 2019;40(13):1081–8.
90. Docherty A, O'Donnell R, Brunskill S, Trivella M, et al. Effect of restrictive versus liberal transfusion strategies on outcomes in patients with cardiovascular disease in a non-cardiac surgery setting: systematic review and meta-analysis. BMJ. 2016;352:i1351.
91. Wilkinson K, Brunskill S, Doree C, Trivella M, et al. Red cell transfusion management for patients undergoing cardiac surgery for congenital heart disease. Cochrane Database Syst Rev. 2014;7(2):CD009752.
92. Desjardins P, Turgeon A, Tremblay M, Lauzier F, et al. Hemoglobin levels and transfusions in neurocritically ill patients: a systematic review of comparative studies. Crit Care. 2012;16(2):R54.
93. Patel N, Murphy G. Transfusion triggers in cardiac surgery: where do we go from here? Can J Anesth. 2018;65(8):868–72.
94. Vincent J, LeLubre C. The sicker the patient, the more likely that transfusion will be beneficial. J Thorac Dis. 2017;9(12):4912–4.
95. Vincent J. We should abandon randomized controlled trials in the intensive care unit. Crit Care Med. 2010;38(10 Suppl):S534–8.
96. Ospina-Tascon G, Buchele G, Vincent J. Multicenter, randomized, controlled trials evaluating mortality in intensive care: doomed to fail? Crit Care Med. 2008;36(4):1311–22.
97. Holst L, Carson J, Perner A. Should red blood cell transfusion be individualized? No. Intensive Care Med. 2015;41(11):1977–9.
98. Rahimi-Levene N, Koren-Michowitz M, Zeldenstein R, Peer V. Lower transfusion trigger is associated with higher mortality in patients hospitalized with pneumonia. Medicine (Baltimore). 2018;97(12):e0192.
99. Shander A, Javidroozi M, Perelman S, Puzio T, Lobel G. From bloodless surgery to patient blood management. Mount Sinai J Med. 2012;79:56–65.
100. Shander A, Gross I, Hill S, Javidroozi M, Sledge S. A new perspective on best transfusion practices. Blood Transfus. 2013;11:193–202.
101. Gooble S, Gallagher T, Gross I, Shander A. Society for the advancement of blood management, administrative and clinical standards for patient blood management programs. 4th edition (pediatric version). Pediatr Anesth. 2019;29(3):231–6.
102. Shander A, Javidroozi M, Lobel G. Patient blood management in the intensive care unit. Transf Med Rev. 2017;31:264–71.
103. Murphy M, Goodnough L. The scientific basis for patient blood management. Transf Clinique et Biol. 2015;22(3):90–6.
104. Franchini M, Munoz M. Towards the implementation of patient blood management across Europe. Blood Transfus. 2017;15(4):292–3.
105. Althoff FC, Neb H, Herrmann E, Trentino KM, Vernich L, Füllenbach C, et al. Multimodal patient blood management program based on a three-pillar strategy: a systematic review and meta-analysis. Ann Surg. 2019;269(5):794–804.
106. Farmer S, Towler S, Leahy M, Hoffmann A. Drivers for change: western Australia Patient Blood Management Program (WA PBMP), World Health Assembly (WHA) and Advisory Committee on Blood Safety and Availability (ACBSA). Best Pract Res Clin Anesthesiol. 2013;27(1):43–58.
107. Fischer D, Schafer S, Jea R, et al. Changes in transfusion practice associated with the introduction of Patient Blood Management. Anesthesiologie Intensivmedizin. 2018;59(5):234–9.
108. Frietsch T, Shander A, Faraoni D, Hardy J-F. Patient Blood Management is not about blood transfusion: it is about patients' outcomes. Blood Transfus. 2019;17:331–3.
109. Clevenger B, Mallett S, Klein A, Richards T. Patient Blood Management to reduce surgical risk. Br J Surg. 2015;102(11):1325–37.
110. Booth C, Allard S. Blood transfusion. Medicine. 2017;45(4):244–50.
111. Desai N, Schofield N, Richards T. Perioperative Patient Blood Management to improve outcomes. Anesth Analg. 2018;127(5):1211–20.
112. Meybohm P, Froessler B, Goodnough L, Klein A, Munoz M, et al. Simplified international recommendations for the implementation of Patient Blood Management. (SIR4PMB). Periop Med. 2017;6:6–13.
113. Mueller M, Van Remoortel H, Meybohm P, Aranko K, et al. Patient Blood Management: recommendations from the 2018 Frankfurt consensus conference. JAMA. 2019;321(10):983–97.

114. Bolcato M, Russo M, Trentino K, Isbister J, et al. Patient Blood Management: the best approach go transfusion medicine risk management. Transf Apheresis Sci. 2020;50(4):102779.
115. Edwards J, Morrison C, Mohiuddin M, Tchatalbachev V, Patel C, Schwickerath VL, et al. Patient blood transfusion management: discharge hemoglobin level as a surrogate marker for red blood cell utilization appropriateness. Transfusion. 2012;52(11):2445–51.
116. Ramsey G, Wagar E, Grimm E, Friedberg R, a. Red blood cell transfusion practices: a College of American Pathologists Q-probes study of compliance with audit criteria in 128 hospitals. Arch Pathol Lab Med. 2015;139(3):351–5.
117. Shander A, Lobel G, Javidroozi M. Transfusion practices and infectious risks. Expert Rev Hematol. 2016;9(6):597–605.
118. Meybohm P, Lindau S, Treskatch S, Spiess C, et al. Liberal transfusion strategy to prevent mortality and anemia-associated ischemic events in elederly non-cardiac surgical patients -- the study design of the LIBERAL trial. Trials. 2019;20:101.

Oxygen and ATP: the Energy Economy of the Cell

2

George P. Biro

"The lighted candle respires, and we call it flame. The body respires and we call it life. Neither flame nor life is substance, but process."

John W. Severinghouse: Foreword to the First Edition, Applied Respiratory Physiology, J. F Nunn, Butterworth and Co., 1969.

Introduction

The theme of this book is the means whereby oxygen is moved from the alveolus to all the cells of the body where it is required for the conversion of foodstuffs to chemical energy, in the form of adenosine triphosphate (ATP). The latter is required for the proper operation of all cellular processes. Accordingly, it seems appropriate to provide a concise summary of how oxygen enables the energy metabolism of the cell. The entire system is highly regulated to assure that sufficient oxygen is provided to assure the proper functioning of all its constituent organs and to permit the organism to meet varied demands [1].

Oxygen

Oxygen is one of the most abundant elements in the earth's crust and is present in the atmosphere in 21%. It was discovered by *Carl Wilhelm Scheele* (1742–1786) and *Joseph Priestley* (1733–1804). The former made the discovery first, but it is attributed to Priestley because his description was published first [2, 3]. *Antoine-Laurier Lavoisier,* (1743–1794), who has been called the "father of modern chemistry, performed trailblazing experimental studies and identified oxygen as the substance key to *both combustion and respiration* [4].

Oxygen (atomic number 8) is a highly reactive element and is present in the atmosphere overwhelmingly in the diatomic, molecular, form of oxygen gas, O_2. The high reactivity of the atomic element is due to having two unpaired electrons in its outer orbit attracting other elements to form many compounds. Oxygen can also form a triatomic form, O_3, ozone, as well as many transient radicals and oxidizing forms, such as hydrogen peroxide, H_2O_2.

For our purposes, the two most important features of molecular oxygen are:

- Its abundance in atmospheric air, and
- Its property as a powerful electron acceptor**.**
- **I**ts abundance in atmospheric air ensures its availability in inspired air and the transport system from the atmosphere to the mitochondria in all living cells.

The process of energy conversion from chemical bonds in glucose is a process of dividing the carbon chain and progressive transfer of high energy electrons ultimately to oxygen as the ultimate electron acceptor in *aerobic respiration*. The latter is the means whereby the energy requirements of all cells are met by the production of *adenosine triphosphate (ATP).* In addition to beneficial properties, oxygen also enters other electron acquisitions that yield unstable radicals and ions.

The utilization of atmospheric oxygen by the organism depends on the *oxygen transport chain* which consists of the following sequential transport processes:

- Cardiac action by the right ventricle maintains constant blood flow through the pulmonary circulation, continuously exposing venous blood to alveolar air, where only the alveolo-capillary barrier permits physical separation, but gas diffusion efficiently driven by the gas partial pressure difference between alveolar air and capillary blood.

G. P. Biro (✉)
University of Ottawa, Ottawa, ON, Canada
e-mail: gbiro@uottawa.ca

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_2

- Pulmonary ventilation whereby alveolar air is continually recycled by mass movement to maintain constant oxygen partial pressure, pO_2, by muscular effort, required to maintain continuous diffusion of oxygen into the pulmonary capillary blood to saturate erythrocytic hemoglobin.
- The alveolar air equation defines the partial pressure of oxygen in alveolar air when the fractional concentration of oxygen in inspired air (F_iO_2) is 0.21, as:

$$pO_2 \text{ in alveolar air} = pO_2 \text{ in inspired air} - \left(pCO_2 \text{ in alveolar air} / \mathrm{R} \right)$$

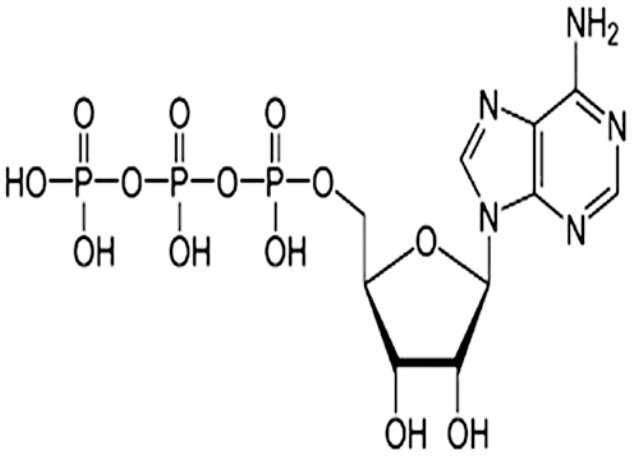
- The pO_2 in alveolar air drives oxygen diffusion into pulmonary capillary blood, as the oxygen dissociation curve of hemoglobin determines the oxygen saturation of blood leaving the pulmonary capillaries.
- Contraction of the left ventricle drives blood flow returning to the left atrium to the aorta and systemic arteries and arterioles, by mass transport.
- Arterial blood pressure and arteriolar resistance in the organs determine the rate of blood flow through each organ and thereby the oxygen flux.
- It is in the microcirculation that oxygen delivery and consumption take place. The architecture of capillary beds of different organs have evolved to subserve the specific organ's function, both to assure appropriate oxygen supply for the organs' metabolic needs, as well as the organs specific function (e.g., absorption, secretion, ion transport, etc.), the capillary bed is regulated to assure optimal blood flow distribution as well as capillary density, whereby diffusion distances can also be optimized.

This complex, efficient and highly regulated system of symmetrical transport processes have evolved to assure optimal oxygen availability to all cells, at a minimal energy cost.

Adenosine Triphosphate

Adenosine triphosphate (ATP) was discovered by *Karl Lohman* (1898–1978) in 1929 as a molecule that was consumed in muscular contractions. The molecule was synthesized by *Alexander Todd* (1907–1997) who received the 1957 Nobel prize. It is one of the essential chemicals in the maintenance of life in all organisms.

The molecule comprises adenine (a nitrogenous base), bound to ribose (a 5-carbon sugar) and three phosphate groups arranged in a linear backbone of covalent phosphorus-oxygen-phosphorus bonds. The terminal, or gamma, phosphate bond is a high energy bond whose energy can be released when the phosphate is stripped away by hydrolysis. It can be generated from adenosine diphosphate by the phosphorylation reaction.[1] Adenosine triphosphate and adenosine diphosphate are in a continuous cycling process.



The molecule is an ancient evolutionary remnant originally evolved and utilized by primordial organisms and conserved in all animal and plant higher organisms. It is used universally as an energy storage form in all energy-dependent cellular processes. It is not a "general storage form" of energy for *eventual* use but is produced and consumed in a *continuous* cycle whose velocity is matched to the prevailing rate of cellular processes, including those of syntheses, membrane-bound ion pumps, contractile mechanisms, and electric charge movement in nerve and muscle cells. The molecule is produced when food stuffs, such as glucose, are broken down into smaller molecules, by a reduction reaction from adenosine diphosphate (ADP) in a series of cellular pathways that will be described below in this chapter.

ATP has been compared functionally to a battery that can be charged or discharged to power a process. It has also been compared to a form of "currency" because it is a uniform entity used and produced by all cells. In a national economy a currency is produced and distributed by a central bank for use by all subordinate units (e.g., municipalities, businesses, consumers) for the purchase of goods and services. In this sense, ATP performs the same function as a singular entity. It is also similar in that it is part of a "supply chain". In a national economy the currency functions as a "just-in-case" used entity. In the economy of the organism and its constituent cells, the ATP "currency" functions as a *"just in time"* availability, bypassing the central bank's role and being produced at the immediate site of utilization within the cell itself thereby contributing to more efficient use.

[1]Phosphorylation is the reaction whereby a high energy phosphate groups is attached to any molecule with removal of a molecule of water (hydrolysis).

The biological oxidation of glucose proceeds in multiple stages, each consisting of several steps to produce energy in a form which can be utilized by the body: ATP. ATP moves within ultra-short distances within the cell from its site of production to its utilization. It is not stored but is produced as the cell's need for energy waxes and wanes [5, 6].

ATP Production: An Overview

ATP is generated in a consecutive series of three distinct metabolic pathways:

- *Glycolysis* (also referred to as *Embden Meyerhof pathway*) whereby the 6-carbon sugar, glucose, is broken down to two 3-carbon fragments.
- *Krebs cycle* (also known as *citric acid cycle*) whereby the 3-carbon pyruvate is cleaved to a 2-carbon molecule with the release of carbon dioxide. As well, high energy intermediates are produced which carry an excess of high energy electrons.
- *Electron transport chain* (E.T.C.) (also known as *oxidative phosphorylation*) whereby high energy electrons are transferred enzymatically from electron donors to electron acceptors, while protons are transported across the mitochondrial membrane.

ATP is generated by all three pathways in varying yield, as well as high energy intermediates,[2] nicotinamide adenine dinucleotide, NAD and flavin adenine dinucleotide, FAD that are reduced to $NADH^{-}$and $FADH^{2-}$.

Oxygen Utilization: An Overview

In the covalent bonds in foodstuffs, e.g., glucose, chemical energy is trapped which can be liberated by enzymatic breaking of C-C, C-O and C-H bonds. Enzymatic action ensures that energy liberated is not "wasted" but is transferred efficiently to other chemical entities' covalent bonds, although all such actions are also accompanied by the production of heat. The dominant *energy storage form is ATP.* The progressive breakdown of larger molecules (e.g., glucose) is maintained only when, in the *final stage* of the sequence of three metabolic pathways, *oxygen is available.*

Molecular oxygen is delivered by the "oxygen transport system" from the atmosphere by the sequence of: alveolar ventilation, diffusion across the alveolo-capillary membrane, mass transport through the circulation by cardiac contraction and, finally by diffusion in the microcirculation from capillary blood to the cells, and diffusion within the cells to the site of ultimate utilization in the mitochondria [7]{Pittman, 2013 #259}. As excess high energy electrons are delivered by the intermediates $NADH^{-}$ and $FADH_2^{2-}$ to the mitochondria where a series of membrane bound complexes deliver these high energy electrons to the *ATP synthase enzyme* where electrons and protons are transferred to oxygen in the reaction sequence:

$$4\,\mathrm{NADH}^{-} \rightarrow NAD + \mathrm{O}_2 \rightarrow \mathrm{H}_2\mathrm{O} + \text{chemical energy}$$

$$\mathrm{FADH}_2^{2-} \rightarrow \mathrm{FAD} + \mathrm{O}_2 \rightarrow \mathrm{H}_2\mathrm{O} + \text{chemical energy}$$

$$ADP + \mathrm{P}_i + \text{energy} \rightarrow ATP + \text{heat.}\left[8-10\right]$$

The Cell's Energy Economy

Aerobic Respiration

Three consecutive metabolic pathways produce ATP in each cell [1].

Glycolysis

Glycolysis [11, 12] is the first series of reactions whereby glucose ($C_6H_{12}O_6$) is cleaved to two three-carbon compounds. Glucose first enters the cell through a membrane bound transporter and then undergoes a ten-step process within the *cytosol*. Of the ten steps the first five involve the preparation for the second, "productive" five steps. The former involves some trivial molecular rearrangements (isomerization to fructose) and the "investment" of *two* ATP molecules to *phosphorylate*[3] the six-carbon sugar, at its two "ends" to yield *fructose-1,6-diphosphate*. In this form the sugar is trapped within the cytosol. In the second five steps the six-carbon phosphorylated sugar is cleaved between the third and fourth C-C bonds to yield *two* three-carbon moieties, each with an attached terminal high energy phosphate. The cleavage of the six-carbon sugar also yields energy that is used in the process of "substrate level phosphorylation" adding an ATP to each product. Unlike the initial "investment" of an ATP from the cell's store, this uses ADP and the energy from the cleavage. The cleavage yields two three-carbon compounds, *1,3 diphosphoglycerate and phosphoenol pyruvate* [12]. Note that these are two "diphosphate" compounds. We have "invested" only two ATP molecules to phosphorylate glucose but have two products, each with two high-energy phosphates, a total of four ATP. The final two products are *1,3 diphospho-glycerate* (1,3 DPG) and *phosphoenol pyruvate*. Two high energy phos-

[2]Derivatives of B vitamins Thiamine and Riboflavin.

[3]Phosphorylation is the process whereby a third phosphate group is attached to the second phosphate group by a high energy bond.

phates are stripped from each of the two diphosphate compounds yielding four ATP's.

Thus, *the balance* is the investment of two ATP's and a yield of four ATP's for a *net* yield of *two ATP's for each glucose metabolized*. The *final* product of glycolysis is *pyruvate* that will enter the Krebs cycle.

Coincidentally, in addition to the breaking of the central carbon-carbon bond of glucose, hydrogen is also being stripped from it. Because of the high energy content of the H-C bonds being split, the hydrogen liberated is in the form of "*hydride ion"* which contains two *electrons, H^{2-}, one of which occupies a high energy orbital***.** These high-energy electrons are transferred to the electron acceptors, *NAD, and FAD*, to produce *$FADH^{2-}$ and $NADH^{-}$* and will be transferred to the next phase of aerobic metabolic processes. Ultimately the electrons will be transferred to oxygen in the *final* phase of energy metabolism. If oxygen is not available in that phase the accumulation of high energy nucleotides inhibits further reactions [12].

At this point only a small fraction of the energy content of glucose has been extracted. Further energy extraction occurs in the next series of reactions in the *Krebs cycle*.

Coincidentally, unused glucose is taken up by muscle and the liver and is stored as *glycogen* after a polymerization process. It is then stored and made available when needed.

In Summary

Glycolysis produces a net two ATP molecules for each molecule of glucose used. Glycolysis is but the "introductory" process to the second consecutive series of metabolic steps, the Krebs, or citric acid, cycle.

Oxygen is needed at the very end of the series of reactions to complete the entire sequence to permit it to run to its conclusion and to permit the entry of pyruvate into the Krebs cycle. In the absence of oxygen, the Krebs cycle grinds to a halt, and pyruvate is converted to lactate, which is a blind-end product, most prominent in muscle during exercise when oxygen availability is limiting. This leads to the concept of *oxygen debt*. The reaction is catalyzed by the enzyme lactate dehydrogenase:

$$\mathrm{PYRUVATE} + \mathrm{NADH}^{-} + \mathrm{H}^{+} \rightarrow \mathrm{LACTATE} + NAD^{+}$$

Note that this conversion is using one $NADH^{-}$ produced above, since it cannot continue in the electron transfer process. And the reaction can be reversed when oxygen is available, "repaying" the oxygen debt. Some of the lactate produced during intensive muscular activity is converted back to glucose by the liver.

In clinical situations of *hypoxia,* lactate accumulates in cells and spills into the blood plasma; an elevated plasma lactate concentration is a clinical indicator of *hypoxia*, oxygen deficiency. In such cases we are dealing with *ANAEROBIC RESPIRATION* [12].

Exceptional Case: Glycolysis in the Erythrocyte

The mature erythrocyte is a prominent example of molecular evolution, as it has adapted to its principal function, namely the most efficient transport of oxygen.[4] To that end, it has lost most of its organelles, including the nucleus and the mitochondria, to enable a high concentration of hemoglobin. Also, it does not use oxygen in its metabolism. Its metabolic apparatus is functionally concentrated on three aspects:

- the protection of heme iron from oxidation.
- the prevention of denaturation of the globin by the hexose monophosphate shunt.
- the regulation of oxygen affinity of hemoglobin by allosteric modification by 2,3 diphosphoglycerate (2,3 DPG).

The erythrocyte contains a modified complement of the glycolytic apparatus. The entry of glucose in the erythrocyte does not require facilitation by insulin. Glucose undergoes glycolysis but with a detour in that 2,3 diphospho-glycerate is produced in high yield by a *mutase* reaction. 2,3 DPG functions as an allosteric modulator of hemoglobin oxygen affinity: a high concentration results in lowering the oxygen affinity, whereas a low concentration increases oxygen affinity. Two inherited metabolic enzyme deficiencies are hexokinase and pyruvate kinase deficiencies with corresponding failure to modulate hemoglobin oxygen affinity [8]. The erythrocyte is continuously exposed to a high concentration of oxygen favoring the production of oxidants and denaturation of globin. Reduced glutathione functions as a major antioxidant in protecting against oxidant damage. The oxidation of heme iron ($Fe^{2+} \rightarrow Fe^{3+}$) is reversed by methemoglobin reductase enzyme, using $NADH^{-}$ [8].

The Krebs Cycle

In the next phase of aerobic metabolism, the end-product of glycolysis, *pyruvate* feeds into the *Krebs, or tricarboxylic cycle*.

Hans Adolph Krebs (1900–1981), together with Fritz Lipman (1899–1986), elucidated the chemical reactions in the cycle that follows glycolysis in the sequence of energy production and for which they received the Nobel Prize in 1953.[5]

The link between glycolysis and the Krebs cycle is the irreversible "oxidative decarboxylation" reaction: Pyruvate, the end-product of glycolysis, reacts with Coenzyme A:

$$\mathrm{Pyruvate} + \mathrm{Coenzyme\ A} + NAD^{+} \rightarrow \mathrm{acetyl} - CoA + CO_2 + \mathrm{NADH}.$$

[4] This is explored extensively in Chaps. 3 and 4 of this book.

[5] Sir Hans Krebs elucidated the citric acid cycle, the glyoxalic cycle, invented the Krebs Henseleit solution for perfusion of isolated organs. His work commenced at the University of Sheffield, then moved to Oxford University where a laboratory is named in his honor.

Unlike glycolysis which takes place in the cytosol, the *Krebs cycle reactions take place in the mitochondrial matrix*. The mitochondria are constituent organelles of all cells and are composed of an outer membrane, an inner membrane which is extended in its length by infoldings called cristae, an intermembrane space and the matrix which is encased by the inner membrane. Enzymes are embedded in the membranes and other reactions occur in the matrix.

Coenzyme A is ubiquitous in metabolism, the universal carrier of the two-carbon acyl groups. It was discovered by Fritz Lippman as the factor involved in the attachment of acetyl groups. Its reactive site is the terminal sulfhydryl (-SH group). It is functionally like ATP in that it acts as a carrier of high energy acyl groups. Its hydrolysis is energetically favored:

$$\text{acetyl} - CoA + H_2O \rightarrow \text{acetate} + CoA$$
$$+H^+ + \text{chemical energy} + \text{heat}.$$

Note that glucose is not the only source of two-carbon compounds in metabolism. Two-carbon fatty acid fragments, the products of β-oxidation of longer chain fatty acids, and some amino acids can also enter the Krebs cycle.

Overview

The Krebs cycle is a closed circle that begins with acetyl-CoA reacting with the four-carbon molecule, oxaloacetate. The CoA moiety is then split off, yielding the six-carbon citrate molecule. One turn of the cycle comprises eight consecutive reaction steps that *yield a total of three NADH⁻, one FADH⁻, two molecules of CO_2 is released and one molecule of ATP or GTP is generated.* The latter is a guanosine analogue of ATP, used in some cells to power transcription in protein synthesis. Note that the production of CO_2 in the Krebs cycle is entirely separate from the consumption of O_2 by oxidative phosphorylation, in the third series of reactions [12].

Some Details

The beginning of the cycle involves the combination of oxaloacetate and acetyl CoA, followed by cleavage off the CoA and the six-carbon chain, citrate, is formed (hence the alternative name of citric acid cycle). Following trivial internal rearrangements (isomerization), two carbons are released as CO_2 with one $NADH^-$ produced each time. The remaining four-carbon molecule, α-ketoglutarate, is extended by reacting with another acetyl-CoA to form the six-carbon succinyl-CoA.[6] The CoA is again cleaved off to yield the six-carbon succinate and subsequent loss of two carbons as CO_2. The four-carbon intermediates end up with oxaloacetate (C-4), the starting product of the next turn of the cycle. Of the eight steps of the cycle, five are productive of some utilizable entities, ATP and $NADH^-$ or $FADH_2^{2-}$.

Note that the original starting substrate was glucose which was split to two glycolysis products that entered the cycle from the original glucose molecule.

Thus, *the net yield of one glucose molecule* through glycolysis and the Krebs cycle are:

Two ATP or GTP, eight *$NADH^-$, two $FADH_2^{2-}$ and the release of six molecules of CO_2.*

The velocity of the Krebs cycle is regulated by critical enzymes, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, according to the prevailing demands for energy by the individual cell.

Thus far, the energy economy has not yet been highly productive of ATP. The final series of reactions, the "*electron transport chain" (ETC), also called "oxidative phosphorylation",* will generate the overwhelming majority of ATP's from the progressive enzymatic transfer of high energy electrons and protons to the ultimate electron acceptor, O_2.

The Electron Transport Chain, Oxidative Phosphorylation

All roads lead to Rome: the final common pathway of ATP production.

Oxygen plays its vital role in the third and terminal stage of reactions. Unlike in the Krebs cycle reactions which take place in the mitochondrial matrix, the Electron Transport Chain (ETC) reactions are catalyzed by the enzyme complexes embedded in the mitochondrial inner membrane.

Note that the three phases of aerobic metabolism are not only distinct processes but are physically localized in different parts of the cell. Nevertheless, from the economic perspective, all ATP is generated within the individual cell that utilizes it. There is no central bank producing and distributing currency to end-users.

The membrane bound enzyme complexes are arranged in physical sequence and include the cytochromes. The electrons in $NADH^-$ generated above, are carried along from complex to the neighboring complex, ultimately reaching the terminal cytochrome c_3 where the reaction of two high energy electrons, two protons and one oxygen atom react to form water [12]. For each half molecule of oxygen three molecules of ATP are produced. This is referred to the *oxidation state of P/O = 3.*

From *one molecule of glucose* oxidative phosphorylation produces thirty-two molecules of ATP. Thus, in total, one molecule of glucose through biological oxidation generates 32 + two + two = *36 molecules of ATP*.

In vitro physical *combustion* of one mole of glucose liberates 2820 kJoule of energy as heat. *Biological oxidation* of one mole through *aerobic metabolism* generates 36 moles of ATP, the equivalent of 1270 kJoule of usable energy as high energy compounds. *Anaerobic metabolism* of one mole of

[6] Succinyl-CoA is also used in the synthesis of porphyrins.

glucose produces lactate and only two moles of ATP, representing only 67 kJoule of usable energy. Thus, the delivery of oxygen in sufficient quantities to the mitochondria permits an energy efficiency of about forty-five percent. This compares favorably to nearly all man-made machinery. The rest is in heat maintaining a constant body temperature [5].

Mitochondria; Friend or Foe?

The acquisition of mitochondria during evolution by eukaryotic organisms has been revolutionary, permitting the production of 36 molecules of ATP from a single six-carbon sugar molecule by means of glycolysis, the Krebs cycle and oxidative phosphorylation, instead of just two by glycolysis alone [13]. The mitochondria are strange beasts, indeed; they are foreign acquisitions Their double membrane, their own genome, and transcriptional and translational apparatus are nothing like our own. But they represent one of the most successful symbiotic arrangements in nature. They permit us to fight, flee, run marathons, and keep warm. Beside their ability of utilizing oxygen, benefitting the host, there is still a price to pay for their collaboration: they also harbor the means to make *reactive oxygen species* (ROS). Even this downside is exploited in immune reactions and other functions [13]. This property of generating unstable radicals is discussed in section "Oxygen Sensing", below.

The Energy Economy of Vital Organs

The total oxygen consumption of the mythical 70 kg person at rest is approximately 250 mL/min. This is distributed among all the organs according to their need by a variety of regulatory mechanisms modulating the regional distribution of the cardiac output. The latter at rest is approximately 5000 mL/min. Under maximal exertion the oxygen consumption may increase about 12-fold, while the maximal attainable increase in cardiac output is about five-fold. This clearly implies that under such circumstances, a major redistribution of the cardiac output must occur, in addition increased oxygen extraction from the blood delivered to the tissues.

Note that in the following section how the organ's functions and energy requirements are reflected in their blood flow regulation and oxygen supply. Furthermore, as demonstrated in an excellent study, the severity of dilutional anemia at which tissue hypoxia first appears, as indicated by increased HIF and NOS expression, varies between organs [14].

The Heart

The heart comprises overwhelmingly a single cell type, the myocyte. These cells provide the contractile energy to generate pressure and flow. While comprising of the same cell type, the left and right ventricles differ in the pressure against which they work but generate the same flow. Accordingly, the two ventricles differ in their mass and volume; left ventricular mass is three to four times that of the right. The two exceptions, comprising a vanishingly small proportion of the heart mass, the endothelial lining (endocardium) and the specialized pacemaker and conducting cells, have a much smaller energy requirement than contractile myocytes [15].

The heart is continually active generating tension in its walls thereby creating pressure and flow. This continual activity requires a continuous supply of energy for the operation of myofibrillar ATPase activity. This energy demand must be satisfied by the delivery of oxygenated blood through the coronary circulation. The oxygen consumption of the working heart while the body is at rest is 9–10 mL/100 g, or about 27–30 mL/min. on average. The oxygen consumption of the arrested perfused heart is about 2 mL/min. Thus, the actual contractile work of the heart requires four to five times more oxygen than its basal metabolism [16, 17].

The primary metabolic substrate of the working heart is not glucose, but fatty acid fragments derived from β-oxidation of long chain fatty acids. During transition from one level of workload to another, cardiac glycogen stores also contribute metabolic substrate [16]. The heart's substrate metabolism comprises an "intricate set of pathways that result in both ATP producing and non-ATP producing endpoints for each class of substrates" [18].

The heart is unique in that its oxygen consumption is highest among all organs at rest, except for the carotid body chemoreceptors. It is also an obligate aerobic organ requiring constant and continuous supply of oxygen by sufficient blood flow through the coronary circulation [16]. Its workload can vary several-fold when the body requires enhanced oxygen delivery, for example during vigorous exertion. The coronary circulation is also unique in that it has the highest rate of oxygen extraction from its blood flow, limiting its ability to enhance oxygen supply by greater oxygen extraction. Hence, the coronary vascular bed must be capable of increasing blood flow by up to five-fold to meet highly variable demands. In addition, the contraction-relaxation cycling of the left ventricle imposes extra limitations on its perfusion. During the phase of contraction, systole, the intramyocardial smaller coronary arteries are compressed thereby limiting their blood flow. Hence, most of the perfusion of the myocardium of the left ventricle is limited to the phase of relaxation, diastole, when the intra-myocardial branches are not compressed. As the heart meets increased systemic demands, the heart rate accelerates and thereby the duration of each cardiac cycle is shortened and the diastolic period, during which most of the perfusion occurs, is also shortened. Thus, the coronary circulation must be tightly regulated to meet increased oxygen demands. This is accomplished by a

complex regulatory mechanism in which the release of adenosine plays an important role [9].

One further unique property of the heart is its ability to vary its *contractility.* This is a property that enables the myocardium to generate greater force from any given initial sarcomere length, and to do so at faster velocity of shortening. This can be induced by enhanced sympathetic activity, inotropic drugs, such as digitalis glycosides or β-adrenergic agonists, such as adrenalin and dopamine. An increase in contractility is also accompanied by enhanced contractile work, requiring greater turnover of ATP and a *pari passu* need for increased blood flow. In the heart another high energy compound, *creatine phosphate* (CP)[7] also plays an important role. It functions as another high energy pool capable of replenishing ATP during rapid transitions of workload, analogous to the economy to a "cash card" of low limit.

The energy metabolism of the heart serves its highly variable contractile work. The normal coronary blood flow is tightly correlated with the heart's oxygen consumption and workload.

In view of the heart's absolute dependence of oxygen delivery sufficient to meet its varied workload, the integrity of the coronary vasculature is also essential. The pathology of coronary artery disease introduces a vulnerability for temporary dysfunction (acute ischemic attacks), or even permanent cell damage (infarction).

Electron microscopic images of the myocardium provide insight into how the cellular architecture subserves the cells' energetics [2]. The important functional structures are the contractile apparatus, actin and myosin strands, the sarcoplasmic reticulum (SR) which stores calcium ions, the plentiful mitochondria in intimate proximity to the contractile apparatus, and the intercellular connections that transmit depolarization of the sarcolemma. The latter initiates two processes, the rapid release of massive amounts of calcium, as well as the sarcolemma becoming permeable to calcium. Thus, intracellular calcium concentration will increase instantaneously by one to two orders of magnitude. This *initiates* the contraction by activating actin and myosin. The process is terminated and reversed by active uptake of calcium into the SR and pumping out of calcium outside the sarcolemma. The resulting massive fall of intracellular Ca^{++} concentration stops actin-myosin interactions and breaking of actomyosin complex. Large amounts of ATP are consumed in the myocyte in two processes: the breaking of actomyosin bonds and the active pumping of Ca^{++} back into the SR and to the extracellular space. Hence, insufficient ATP available results in maintained actomyosin bonds, contracture, and very high intracytoplasmic Ca^{++} concentration, with resultant deleterious consequences for the mitochondria.

[7]Also called phosphocreatine.

The mitochondria in cardiac muscle are only second to the contractile apparatus, amounting to about one-third of the total cell volume. Despite the proximity between the contractile elements, the mitochondrial outer membrane is impermeable to the large and charged ATP and ADP molecules. Translocase enzymes are required to ensure ATP supply to the contractile elements.

In summary, the supply of ATP in the myocyte is distributed among the following processes:

- Contraction, "myosin ATPase": approximately 60–70% at rest; more during increased workload.
- Active ion pumping (Ca^{++} and Na^{+} K^{+} Pumps): approximately 10–20%.
- Synthetic processes, action potential, cyclic AMP formation (signaling), vasodilation (adenosine replacement): etc. 10–20%.

The heart cannot tolerate a shortage or absence of oxygen. Lack of blood flow, *ischemia*, either global or regional (as in a coronary occlusion), will interrupt its energy production and contractile activity will rapidly cease within minutes. Continuing ischemia results in reduction of its energy requirements, but failure of ion pumps and a chain of deleterious events will follow that result in death of the ischemic myocytes within hours.

The Brain

Unlike the heart with its unitary cell population and singular function, the brain is different in its vascular anatomy, function, cell types, architecture, and distribution of its energy metabolism. The vast complexity of the functions performed by the various anatomical structures of the brain preclude any detailed discussion.

The brain comprises a large variety of distinct anatomical regions with distinct functions. Fundamentally, it comprises two cell types of neurons and astrocytes. The neural structures subserve a variety of regulatory "autonomic" functions, such as ventilation, circulation, splanchnic organs regulation, etc. These occur as if in the "background". "Higher" structures subserve the cognitive, memory, and adaptive roles, such as plasticity. The most important functions of neurons are those related to synaptic transmission initiated by membrane depolarization. Underlying these functions are changes in membrane potential governed by ion fluxes (Na^{+}, K^{+} pumps) and the synthesis, release, uptake, and resynthesis of a variety of neurotransmitters, or "chemical messengers" (noradrenalin, glutamate, dopamine, histamine, etc.) [18]. While these are functional building blocks of the varied functions performed by the brain, they occur in distinct structural entities. As different functions take place at different times, the energy requirements of these entities are also dynamic [10]. Cerebral blood flow, glucose con-

sumption and oxygen metabolism are all increased in localized structures, in response to a range of activities.

Current research identifies two distinct mechanisms of regulation:

- Neurovascular coupling and
- Neurometabolic coupling.

Neurovascular Coupling

The exact mechanisms underlying the matching of blood flow to neural activity is still unclear; the leading candidate as the mediator of vasodilation is the *nitric oxide radical* ($NO^{\cdot}$). NO in the brain is generated by *neuronal nitric oxide synthase* (nNOS). This, in turn, is stimulated by glutaminergic excitation. Vasodilation (i.e., relaxation of vascular smooth muscle) is mediated by the action of *soluble guanylate cyclase* enzyme (sGC) generating cyclic guanylate monophosphate (cGMP) [19]. NO-mediated reactions are common in physiology and are of great importance in the central nervous system [10, 19]. In addition to promoting vasodilation to match supply to active regions to match oxygen demand, it also has several other roles in communications among neurons and, sometimes deleterious role in neurodegeneration [19].

Note the difference in the vasodilator mediators between heart and brain: adenosine in the former, NO in the latter.

Neurometabolic Coupling

In neurometabolic coupling *astrocytes* play a central, synergistic role with neurons [10]. The former cells are localized in close apposition to both the endothelial wall of capillaries and the synaptic clefts of the neurons. "One of the best characterized functions of the astrocytes in neuronal activation is the maintenance of neurotransmitter stores in the cycling of release, reuptake and repeat release of glutamate" [10]. There is an interesting dichotomy between astrocyte and neuron energy metabolism. Glucose is utilized by astrocytes to produce lactate by glycolysis during activity while neurons utilize the lactate as well the Krebs cycle to satisfy their energy needs. This segregation of energy producing pathways are accompanied, or are mediated, by differential gene expression [10].

The complex architecture and the distribution of different neural functions makes precise localization of oxygenation state during different activity states is hindered by the lack of high resolution of regional oxygen concentration by functional Magnetic Resonance Imaging (fMRI).

Oxidative metabolism of brain tissue is extremely sensitive to hypoxia. Compromise of neuronal function occurs within minutes of interruption of blood supply. Lesser degrees of hypoxia, of a more chronic nature, induce changes in gene expression of Hypoxia Inducible Factors (HIF's) which, in their turn, modulate transcription. There are several different isoforms of HIF's and these are expressed differentially in different parts of the brain [10].

There is one biologically important negative property of oxygen, namely, its predilection to produce *free radicals*, or reactive oxygen species (ROS), such as hydroxyl and superoxide anions and hydrogen peroxide. These are powerful oxidizing agents that attack proteins and membrane lipids. Neurons are highly vulnerable to oxidative damage by ROS but are protected by the presence of reducing agents, such as glutathione, NADH, as well HIF species [10, 19]. The property of oxygen producing ROS is discussed below.

The Kidney

The kidney differs from both heart and brain in its unique vascular architecture, its excretory functions directed at maintaining the volume, osmolarity and ionic composition of the extracellular fluid, its endocrine products regulating its own function, blood pressure and erythropoiesis. These are all energy intensive activities, and its blood flow and intrarenal flow *distribution* is regulated to assure continuing function responding to changes in body volumes and chemistry. Additionally, blood flow to the kidney is greater than its own metabolic requirement because it is coupled to its excretory functions. Whereas the two kidneys comprise approximatively 0.5% of the total body weight they receive about 25% of the cardiac output.

The basic renal excretory functional unit, the nephron, comprises the following [20]:

- The efferent arteriole provides large blood flow to the glomerulus.
- Transit through the glomerulus where the plasma is filtered.
- The ultrafiltrate within Bowman's capsule contains water and all small molecular weight compounds and ionic constituents of the plasma in the same concentration as plasma.
- The ultrafiltrate enters the proximal convoluted tubule and then the descending limb of Henle which plunges down into the renal medulla.
- The entire loop is surrounded by an extensive capillary network in intimate contact with the loop itself.
- The epithelial cells contain several ion pumps capable of transporting specific ionic species across the epithelium and the capillary endothelium in the tubules, against a concentration gradient.
- This creates an increasing osmotic gradient such that at the turn of the loop interstitial osmolarity is four times (1200 mOsm) that of the glomerular filtrate (300 mOsm)

The largest proportion of energy consumed by the kidney involves the reabsorption of sodium by the specialized proximal tubular cells which contain many mitochondria, in con-

trast to those of Henle's loop whose mitochondrial complement is small [20]. This suggests that the greatest energy expenditure occurs in the former part of the nephron. This part of the nephron is in the outer cortex of the kidney. The renal cortical and medullary blood flows are regulated independently by several vasoactive substances (angiotensin, vasopressin, prostanoids, etc.) [21, 22].

In addition to energy consuming (ATPase) pumps, the kidney also has an important endocrine function. The kidney responds to hypoxia by secreting *erythropoietin*, a hormone that stimulates proliferation of erythroid precursors in the bone marrow, taking approximately 7 days for release of immature nucleated erythrocytes ("reticulocytes") into the circulation that subsequently lose their nuclei and mature in about 3–4 days. The synthetic activity can be enhanced by up to a hundred-fold over basal in response to anemia [20]. This synthetic activity is also an energy cost, though its relative magnitude is not clear.

A second product of the kidney is *converting enzyme* involved in the activation of the polypeptides vasoconstrictor angiotensin and the vasodilator bradykinin.

The Liver

The liver performs a great variety of vital functions. Its architecture is again different from the organs above. It receives a total of about 25% of the cardiac output whereas it is only about 2.5% of total body mass. Its total blood flow amounts to about 100–130 ml/min per 100 g of liver mass [23]. This reflects the fact that one of its principal functions is biochemical processing of foodstuffs absorbed from the intestinal tract. In addition, it also detoxifies toxic substances, metabolizes drugs, stores several entities, such as iron and glucose (in glycogen). Its blood supply is dual in that it is supplied by both the portal vein and the hepatic artery. The former has already passed through the circulation of the intestinal tract, and its oxygen content has already been reduced by extraction through the splanchnic bed and contains modified food stuffs and it is at substantially reduced pressure [23]. The contribution of the portal vein to the total flow received is about two third. The two blood streams enter concurrently the functional units, the acini. Oxygenation is mainly by the hepatic arterial contribution.

The architecture of the liver is a sinusoidal one whereby there is a large blood pool surrounding and perfusing the functional unit, the acinus where the two streams of blood join. The hepatocytes are surrounded by blood, so that continuous and intimate exchanges occur. Blood flow through the liver is normally held constant, in contrast to that of the heart. Within functional units there is a preferential distribution of blood flow such that the most oxygenated blood reaches the central part of the acinus first, where the highest concentration of respiratory enzymes is located, and the highest metabolic activity takes place [23]. Whereas the hepatocytes are morphologically identical, there may be large differences in their metabolic activity even in adjacent cells. Thus, the manyfold functions of the liver can be conceived as being analogous to "multitasking". This may be determined by local oxygen gradients [23].

Hepatocytes located in the periportal zone mainly contain the Krebs cycle and the Electron Transport Chain enzymes, whereas glycolytic enzymes are localized in cells where oxygenation is less intense.

Oxygen Sensing

Regulated systems require feedback information on their functioning. In consumer economics the balance between supply and demand is indicated by the price of a commodity. In the physiological energy economy two indicators operate. The carotid and aortic body chemoreceptors on the organism level and the Hypoxia Inducible Factors (HIFs) on the cellular, tissue and organ levels.[8]

Prabhakar and Semenza [24] published an extensive review of the mechanisms of oxygen sensing and their role in homeostasis.

The Carotid Body Chemoreceptors

These are a pair of small structures located in intimate proximity to the carotid sinus baroreceptors, at the bifurcation of the common carotid arteries forming the internal and external carotid branches. Their single function is to "sense" the oxygen partial pressure (pO_2) in arterial blood perfusing them. Their strategic location, sensing oxygenation of the blood on its way to the brain indicates their homeostatic importance. The information from these paired organs is relayed by an afferent nerve, part of the ninth cranial or, glossopharyngeal, nerve to the medullary and pontine respiratory and the medullary cardiovascular centers. Nerve traffic in this nerve is minimal when the arterial oxygen partial pressure is in the normal range of about 100 mmHg. Frequency of nerve traffic increases as the arterial oxygen partial pressure declines, i.e., when alveolar ventilation is not sufficient to saturate hemoglobin. Hypoxia occurs when alveolar ventilation is insufficient, or inspired air is at a lower atmospheric pressure (e.g., high altitude) or when it contains a lower that normal oxygen concentration. The chemoreceptors do not respond to reduced oxygen content due to anemia. The alarm signal from the carotid bodies alerts the brain

[8]The 2019 Nobel Prize in Physiology or Medicine was awarded to W.G Kaelin, Jr., Sir Peter J. Ratcliffe, and G.L. Semenza for the discovery of the Hypoxia Inducible Factors (HIF): https://www.nobelprize.org/prizes/medicine/2019/summary/

stem circulatory and respiratory centers to increase pulmonary ventilation, heart rate and cardiac output and arterial blood pressure [24]. The reflex response, involves activation of the sympathetic nervous system and the nerves mediating respiratory activity (intercostal and phrenic nerves) to increase oxygen supply to organs by the whole oxygen transport system, in addition to some redistribution of the enhanced blood flow to the vital organs by sympathetic and local regulatory responses.

Several lines of evidence indicate that the carotid bodies respond to hypoxia in an exaggerated manner in cardiovascular disease and this may underlie the etiology of "neurogenic" hypertension.

Signal transduction in the carotid bodies makes use of gaseous biochemical messengers, carbon monoxide (CO) and hydrogen sulfide (H_2S).

As discussed above, the various organs utilize organ specific mechanisms and messengers in regulating their individual blood flow and oxygen supply. In particular, the heart and liver depend on adenosine, while the brain utilizes NO-mediated mechanisms. Since the kidneys receive excess blood flow relative to their metabolic needs, it can participate in redistribution for short periods. However, if the danger of hypoxic cellular injury threatens, it releases erythropoietin.

HIF Mediators

These are a family of complex proteins comprising of a variety of subunits that have a dioxygenase activity. This involves the enzymatic incorporation of both oxygen atoms from molecular oxygen in an organic compound to activate or destabilize it. When hypoxic conditions prevail in tissues HIF-1-α can initiate several activities, including erythropoiesis in the bone marrow, angiogenesis, and metabolic adaptation in the hypoxic organ [24].

In critical care practice the state of oxygenation is an important aspect of monitoring since hypoxia can result in cell and organ injury. Several technologies are available for the practitioner [25].

Cardiovascular System and Oxygen Supply Integration: The Role of NO

Haldar and Stamler produced an extensive review of the complex integration of systemic and organ specific supply of oxygen by the cardiovascular system, in which nitric oxide (NO) plays an important role [26]. In the primordial atmosphere NO was abundant, and the biological utilization of oxygen co-evolved with NO. As NO disappeared from the atmosphere, organisms evolved the ability to synthesize NO and use it as a biological signaling molecule. The affinity of NO to cysteine thiols, which are abundant in most proteins, provided the means for modifying target proteins to modify their function. This process is S-nitroxylation. S-nitroxylated proteins are abundant in all organs and particularly so in the cardiovascular system. The NO synthase systems (NOS), abundantly distributed in the organism, respond to changes in oxygen demand and the nitrosylated proteins induce post-translational changes in cardiovascular function. The complexity of this system precludes any further exploration, except to note that there is extensive cross talk among metabolic pathways, ATP generation, regulation of cardiac contractility, mitochondrial function, alveolar ventilation, hemoglobin oxygen affinity and hypoxia-adaptation to high altitude. Dysregulation of S-nitrosylation is thought to underlie cardiovascular disease [26].

In Summary

In chronic hypoxia *Oxygen delivery* can be enhanced by accelerated production and release in the circulation of erythrocytes (erythropoiesis) and the growth of new blood vessels (angiogenesis) whereby the capillary density is increased, decreasing diffusion distances for oxygen.

The consumption of oxygen can also be altered by metabolic reprogramming. This may take place in the interim period when new erythrocytes and capillaries are grown (7–10 days). HIF products are involved in switching gene products resulting in activation of Krebs cycle and cytochrome c enzymes. One strategy some cells may utilize is *cell cycle arrest*, in the face of an imbalance between oxygen supply and demand. This is also mediated by various HIF products.

"Bad" Oxygen Species

The chemical properties of the dioxygen molecule (O_2) are derived from having two unpaired electrons in the outer shell. It may be partially reduced by accepting a single electron that pairs with one of the unpaired electrons, forming a very unstable species, the superoxide anion (O_2^-). This reaction occurs primarily in the mitochondrial respiratory chain [13, 27]. From this species a series of toxic oxygen-derived free radicals, or reactive oxygen species (ROS), can form, including hydrogen peroxide (H_2O_2), the hydroperoxyl radical (H_2O^-) and the hydroxyl radical (OH^-). These are powerful oxidizing agents capable of exacting oxidative damage to protein, DNA, and membrane lipid molecules, causing extensive cellular injury and dysfunction. Several enzymes and antioxidants are also present in most cells that can counteract the deleterious effects of ROS. The degree to which injury results depends on the balance between the rates of ROS production and the antioxidant activity present [5].

ROS are principally produced in the mitochondria in an interconnected network of several metabolic pathways. One way that ROS production may be accelerated is when excess oxygen is present. Thus, cell damage occurring in hyperoxia may be mediated by the excess production of ROS [27]. High levels of ROS, in addition to oxidative injury, may also be involved in immune reactions, causing apoptosis, as well as being involved in disease states [27].

Summary and Conclusions

Evolution has endowed us with a highly efficient transport system of oxygen from the environment to all cells, as well as a utilization system to generate the high energy compounds, ATP and GTP, that all cells need to perform their varied functions optimally. The biochemical pathways of three stages of oxidation and cleavage of the components of food stuffs are adapted to capture and move high energy electrons, derived from the breaking of covalent chemical bonds, in an orderly sequence that minimizes energy loss. This is accomplished by highly regulated and efficient enzyme systems located sequentially within the cytosol, the mitochondrial matrix, and the complexes of the mitochondrial inner membrane, respectively. It is the latter complexes that accomplish the greatest yield of ATP from substrates. Nevertheless, the preceding stages, glycolysis, and the Krebs cycle, are essential to deliver the high energy electrons derived from the stages above, to the Electron Transport Chain Thus, the whole of the sequence is essential to function as a coordinated and well-regulated system.

For optimal cell function a supply chain of energy is required "just in time" and where it is needed, within the cell itself. Thus, the *prevention* of an inadequate energy supply, or *cellular hypoxia* is essential. Cellular hypoxia may arise from failure at any point in the long chain of transport processes from the atmosphere to the mitochondria [7]:

- Reduced atmospheric pressure or oxygen concentration or impaired alveolar ventilation: hypoxic hypoxia.
- Impaired saturation of blood in the lungs or intrapulmonary shunting: hypoxemia.
- Reduced hemoglobin concentration or oxygen combining capacity of the blood: anemic hypoxia.
- Inadequate cardiac output or obstruction in the arterial supply: ischemic hypoxia.
- "Poisoning" of the metabolic pathways: histotoxic hypoxia.

Detection of cell hypoxia, by localized oxygen sensing, and its correction is essential to prevent or ameliorate the consequences of energy starvation that may have important, or even lethal, consequences. The most vulnerable in this regard are the obligate aerobic vital organs, the heart and brain. Their hypoxia tolerance can be measured in minutes or at most a few hours. If uncorrected in time, cell death follows and their ability to regenerate is extremely limited. The kidney and liver are also limited in their hypoxia tolerance but their ability to recover from temporary hypoxic injury is somewhat better that that of heart and brain.

Key Points

- All animal life depends on atmospheric oxygen (O_2) because of its unique chemical properties; because of it high reactivity due to having two unpaired high energy electrons in it outer orbitals.
- All cells have an absolute requirement for readily available biochemical energy in the form of high-energy phosphate bonds in adenosine triphosphate (ATP) and guanosine tri-phosphate (GTP), for the maintenance of all cellular functions.
- High energy phosphate compounds are generated in all cells by a complex series of enzymatic reactions whereby, the energy liberated by the cleavage of carbon-hydrogen bonds in food stuffs is transferred to high energy electrons which, in their turn, are sequentially transferred to intermediate compounds. Some of the intermediate compounds release their energy to phosphorylate adenosine di-phosphate, yielding ATP.
- Three sequential series of reaction steps comprise glycolysis, the Krebs cycle and the Electron Transport Chain (E.T.C.). Each of these yields ATP but the highest ATP yield is provided by the E.T.C. by reacting high-energy electrons with molecular oxygen to yield water (H_2O).
- In the absence of sufficient availability of oxygen all cellular processes may grind to a halt, and if it persists, may lead to irreversible cellular injury. The obligate aerobic organs, heart, brain, kidneys and liver, are most susceptible to hypoxic injury.

Acknowledgement The authors declares no conflict of interest.

References

1. Pittman R. Oxygen transport in the microcirculation and its regulation. Microcirculation. 2013;20(2):117–37.
2. Borman S. Joseph Priestley remembered. Chem Eng News. 2004;2004:41–5.
3. McEvoy J. Joseph Priestley. Encyclopedia Britannica 2020.
4. Donovan A. Antoine Lavoisier. Encyclopedia Britannica 2020.
5. Nunn J. Applied respiratory physiology. Butterworth and Co.; 1987.

6. Zilverberg M, Shorr A. Effect of restrictive transfusion strategy on transfusion-attributable severe acute complications and costs in the US ICUs: a model. BMC Health Serv Res. 2007;7:138–54.
7. Biro G. From the atmosphere to the mitochondria: the oxygen cascade. In: Kim H, Greenburg A, editors. Hemoglobin-based oxygen carriers as red cell substitutes and oxygen therapeutics. Berlin Heidelberg: Springer Verlag; 2013. p. 27–53.
8. Brown K. Erythrocyte metabolism and enzyme defects. Lab Med. 1996;27(5):329333.
9. Duncker D, Bache R. Regulation of coronary blood flow during exercise. Physiol Rev. 2008;88:1086–2006.
10. Watts M, Pocock R, Claudianos C. Brain energy and oxygen metabolism: emerging role in normal function and disease. Front Mol Neurosci. 2018;
11. Blanco A, Blanco G. Abstract. Medical Biochemistry: kindle eBooks.
12. Stryer L. Biochemistry. 2nd ed. San Francisco: W. H. Freeman and Co.; 1981. p. 948.
13. Andreyev A, Kushnareva Y, Murphy A, Starkov A. Mitochondrial ROS metabolism: 10 years later. Biochemistry (Mosc). 2015;80(5):517–31.
14. Tsui A, Marsden P, Mazer C, Sled J, Lee K, Henkelman R, et al. Differential HIF and NOS responses to acute anemia: defining organ-specific hemoglobin thresholds for tissue hypoxia. Am J Physiol Integr Comp Physiol. 2014;307:R13–25.
15. Scheibert H. Anatomy and physiology of coronary blood flow. J Nucl Cardiol. 2010;17(4):545–54.
16. Goodwill A, Dick G, al. e. Regulation of coronary blood flow. Am J Physiol Compr Physiol. 2017;7:321–82.
17. Kiel A, Goodwill A, Barnard A, al. e. Regulation of myocardial oxygen delivery in response to graded reductions in hematocrit: role of K+ channels. Basic Res Cardiol [Internet]. 2017;2019(112):1–14.
18. Kolwicz SC Jr, Purohit S, Tian R. Cardiac metabolism and its interactions with contraction, growth and survival of the cardiomyocyte. Circ Res. 2014;113(5)
19. Knott A, Bossy-Wetzel E. Nitric oxide in health and disease of the nervous system. Antioxid Redox Signal. 2009;11(3):541–53.
20. Koeppen B, Stanton B. Berne and levy physiology. 7th ed. Elsevier Health Sciences; 2017. 944 p.
21. O'Connor AS, Schelling JR. Diabetes and the kidney. Am J Kidney Dis. 2005;46(4):766–73.
22. Hare G, Han K, Leschchyshyn Y, al. e. Potential biomarkers of tissue hypoxia during acute hemodilutional anemia in cardiac surgery: a prospective study to assess tissue hypoxia as a mechanism of organ injury. Can J Anesth. 2018;65:901–13.
23. Lautt W. Chapter 2: Hepatic circulation: physiology and pathophysiology. San Rafael: Morgan and Claypool Life Sciences; 2009.
24. Prabhakar N, Semenza G. Oxygen sensing and homeostasis. Physiology. 2015;30(5):340–8.
25. Schober P, Schwarte L. From system to organ to cell: oxygenation and perfusion measurement in anesthesia and critical care. J Clin Monitor Comput. 2011;26(4):255–65.
26. Haldar S, Stamler J. S-Nitrosylation: integrator of cardiovascular performance and oxygen delivery. J Clin Invest. 2013;123(1):101–10.
27. Yang S, Lian G. ROS and disease: role in metabolism and energy supply. Mol Cell Biochem. 2020;467:1–12.

Physiological Functions of Blood

3

Verghese T. Cherian

Introduction

Circulation of blood is essential for preserving human life. Blood is a connective tissue made up of a variety of cells in a fluidic extracellular matrix. The total volume of blood is approximately 70 ml/kg body weight in a non-obese person. Its viscosity is about 4.5–5.5 times that of water. The main functions of blood can be classified broadly into transport, defense, and homeostasis (Table 3.1). A basic understanding of the production and physiology of the components of blood helps to comprehend the various tasks that it performs.

Hematopoiesis

In the fetus, prior to 6 weeks of gestation, hematopoiesis, or the production of blood cells, occurs in the yolk sac and after that in the liver and the spleen. However, from the seventh month of fetal life onwards and after birth, the bone marrow is the principal site for hematopoiesis [1]. The pluripotent hematopoietic stem cell within the bone marrow differentiates into either the common myeloid progenitor cell or the common lymphoid progenitor cell. The myeloid progenitor cell differentiates into one of four cell lines, thrombopoiesis (thrombocytes), erythropoiesis (erythrocytes), granulopoiesis (basophil, neutrophil and eosinophil) and monocytopoiesis (monocyte, macrophage and myeloid dendritic cell), while the lymphoid progenitor cell undergoes lymphopoiesis (B- lymphocyte, T- lymphocyte, natural killer cell and lymphoid dendritic cell) [2] (Fig. 3.1).

Erythropoiesis has been shown to be regulated by the coordinated action of members of the GATA series of transcription factors. These proteins are encoded by the GATA1 gene present on the X-chromosome. GATA mediates transcriptional regulation via various gene expressions, chromatin modifications, and the binding of GATA factors in human multipotent hematopoietic stem cells, early erythroid progenitors, and erythroid precursors. GATA factors are believed

Table 3.1 Physiological functions of blood

Transport
• To the tissues: Oxygen, Nutrients, Hormones
• From the tissues: Carbon-dioxide, Metabolites
Defense
• Coagulation
• Immune response
• Healing
Homeostasis
• Acid base balance
• Regulating the body temperature
• Balance of body fluids

V. T. Cherian (✉)
Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, PA, USA
e-mail: vcherian@pennstatehealth.psu.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_3

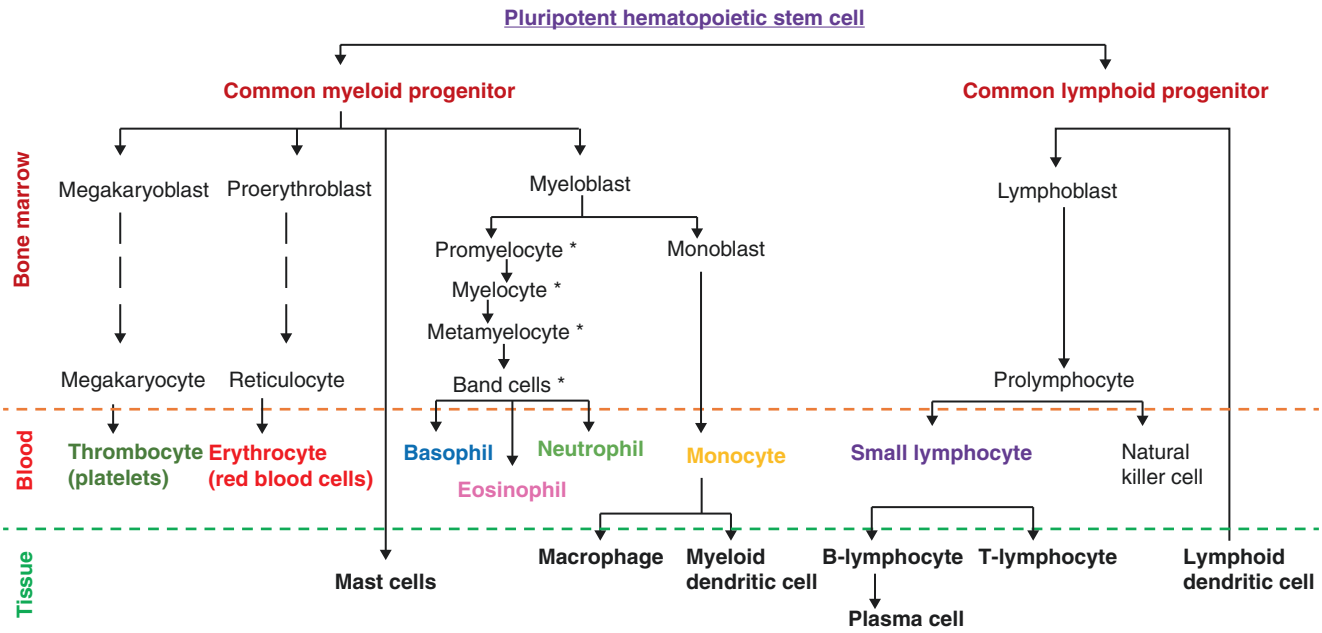


Fig. 3.1 Hematopoiesis. The cells in the blood differentiate from either the common myeloid progenitor cell or the common lymphoid progenitor cell, which is derived from the pluripotent hematopoietic stem cell within the bone marrow. *Although shown as a common lineage for clarity, the promyelocyte, the myelocyte, the metamyelocyte and the band cells for basophil, neutrophil and eosinophil differentiate separately from the myeloblast

to mediate transcriptional changes via a stage-specific interplay with some regulatory elements [3]. The most differentiated erythroblast then extrudes its nucleus and forms the enucleated, hemoglobin-filled reticulocyte. The reticulocyte then migrates into the blood where it matures by shedding other internal organelles, in 1–2 days, to form the 'erythrocyte' or the red blood cells (RBC). The principal factor controlling erythropoiesis is the hormone erythropoietin that is produced, in response to hypoxia, by the interstitial fibroblasts located adjacent to the proximal tubule epithelial cells in the renal cortex. The mature RBC is an 8 μm, biconcave disc, devoid of a nucleus or mitochondria and has a lifespan of 120 days.

Hemoglobin synthesis needs coordination between globin (α, β, γ, δ, ε) gene expression and heme synthesis. Heme synthesis occurs in both the cytosol and the mitochondria of erythroblasts. It begins with glycine and succinyl coenzyme A and ends with the production of a protoporphyrin IX ring, which binds to Fe2+ ion forms the final heme molecule. All the hemoglobin present in the circulating RBC is produced in the developing erythroblast and reticulocyte [4]. There are three types of normal hemoglobin present in the human blood. The fetus primarily produces fetal hemoglobin (HbF), which comprises two α and two γ globin subunits. HbF has a stronger affinity for oxygen which favors flow of oxygen from the maternal to the fetal circulation. Its production drops significantly after birth and accounts to 2–3% of hemoglobin in adults. Hemoglobin A (HbA) is the most common (95–98%) adult form and comprises two α and two β globin subunits. HbA2 is a less common (1–3%) adult form made up of two α and two δ globin subunits. The most common abnormal variant of hemoglobin is HbS (sickle cell hemoglobin), which results from a substitution of glutamic acid with valine at the sixth amino acid position in the β globin subunit. Thalassemia are disorders caused by reductions or absence of globin chain synthesis. Porphyria is a group of disorders caused by defective heme synthesis, due to deficiency of enzymes needed in its production.

Basic Physiology of the Components of Blood

Glucose is the major source of energy for the RBC, and it is anaerobically metabolized by the *Embden-Meyerhof pathway*. Although, two molecules of adenosine triphosphate (ATP) are used in the initial stages, each glucose molecule yields two molecules of glyceraldehyde-3-phosphate molecules and each of which in turn yield two molecules of ATP. Therefore, the Embden-Meyerhof pathway produces two ATP and two reduced nicotinamide adenine dinucleotide (NADH) molecules (Fig. 3.2). The major role of the *hexose monophosphate shunt* is to produce the 'reduced' form of nicotinamide adenine dinucleotide phosphate (NADPH), which is needed to generate the reduced form of glutathione, a major antioxidant within the RBC. Glutathione is essential to protect the cellular enzymes and the hemoglobin from oxi-

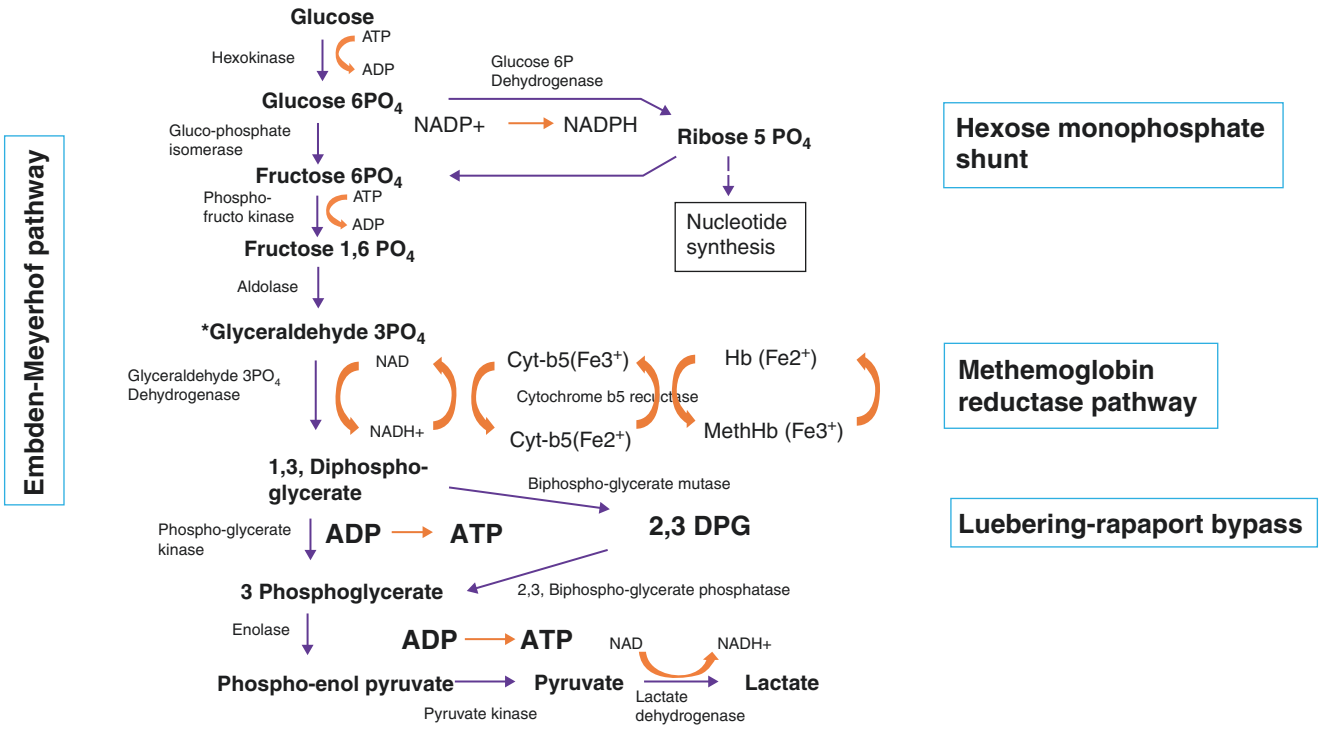


Fig. 3.2 Metabolism in the erythrocyte. Glucose is the major source of energy for the RBC, and it is anaerobically metabolized by the Embden-Meyerhof pathway. (Please refer to the text for the details). ADP adenosine diphosphate, ATP adenosine triphosphate, NADH reduced nicotinamide adenine dinucleotide, NADPH reduced nicotinamide adenine dinucleotide phosphate, 2,3-DPG 2,3-diphospho-glycerate

dant damage. Inadequate glutathione can lead to denaturing of hemoglobin which precipitate as Heinz bodies. The NADP/NADPH levels regulate the amount of glucose metabolized by the hexose monophosphate shunt. Abnormality of this pathway, such as deficiency of glucose-6-phospate dehydrogenase, can lead to hemolysis [5]. An offshoot of the Embden-Meyerhof pathway is the *Luebering-Rapaport bypass*. This pathway provides 2,3-diphosphoglycerate (2,3-DPG), an important regulator of the affinity of hemoglobin to oxygen. The *methemoglobin reductase pathway* uses the enzyme methemoglobin reductase (cytochrome b5 reductase) and NADH to maintain hemoglobin iron in a reduced state (Fe2+).

The white blood cells or the 'leucocytes' can be broadly divided into phagocytes (neutrophils, eosinophils, basophils and monocytes) and lymphocytes. These cells along with an interplay of cytokines, antibodies, and the complement system form the primary immune defense against an infective microbe or a foreign tissue. The platelets or the 'thrombocytes' are non-nucleated cells that are derived by fragmentation from megakaryocytes and survive 10–15 days in circulation. These are crucial for the initiation of blood coagulation and the repair of damaged tissue.

The plasma is an electrolyte solution containing proteins and it forms the vehicle for transport of cells, nutrients, and hormones. It plays a vital role in maintaining the acid-base status, functioning of the immune system, execution of the hemostatic cascade and delivering the cells (fibroblasts) and chemicals needed to heal and repair damaged tissue.

Albumin is the most abundant (3–5 g/dL) protein in the plasma, with a molecular weight of 69 kDalton. It is the significant contributor to the plasma oncotic pressure and forms a vehicle to transport hydrophobic lipids. The second most common plasma proteins are the globulins (1–1.5 g/dL), of which there are three main subgroups: alpha, beta, and gamma globulins. The alpha and beta globulins transport iron, lipids, and fat-soluble vitamins. Immunoglobulins are gamma globulins that form the mainstay of humoral immune response.

Transport of Oxygen and Carbon Dioxide

Oxygen (O_2) is transported from the pulmonary alveoli to the tissues either bound to hemoglobin or dissolved in plasma. Each RBC contains approximately 300 million molecules of hemoglobin (Hb) and each molecule can combine with up to 4 molecules of O_2. The amount of O_2 that binds to the Hb depends on the partial pressure of oxygen (PO_2). At the alveoli, where the PO_2 is about 100 mmHg, the Hb is fully saturated. Although, 1gm of Hb can theoretically combine with 1.39 ml of O_2, in vivo this is about 1.34 ml due to the pres-

ence of other forms of Hb such as methemoglobin and carboxyhemoglobin. When the first molecule of O_2 is bound to the Hb, the conformation of the globin chain changes promoting ease of binding of the subsequent molecules of O_2. This 'cooperativity' is responsible for the sigmoid shape of the 'oxy-hemoglobin dissociation' curve. At the tissue level, where the PO_2 is lower and as a molecule of O_2 is released, the β chain move apart allowing 2,3 DPG to slide in between and lower the affinity of hemoglobin to O_2 and promoting release of O_2. The other factors that reduce the affinity of Hb to O_2 are increasing temperature, acidosis and increased PCO_2 (Bohr effect). The PO_2 at which HbA is 50% saturated (P50) is normally 3.55 kPa (26.6 mmHg). Factors that reduce the affinity of Hb to O_2 increase the P50 or shift the curve to the right while hypothermia, alkalosis and decreased PCO_2 increase the affinity and move it to the left. Carbon-monoxide (CO) affects hemoglobin-oxygen carriage in two ways. Hemoglobin has 200–300 times more affinity for CO than for O2 and carboxyhemoglobin also increases the affinity of the remaining hemoglobin to oxygen (decreases P50), making it less available in the peripheral tissues. Fetal hemoglobin has a higher affinity for oxygen compared to adult hemoglobin (P50 -19 mmHg).

The volume of O_2 dissolved in plasma also depends on the PO_2 and is about 0.003 ml/100 ml/mmHg PO_2. In the arterial blood (P_aO_2 100 mmHg) this translates to 0.3 ml/100 ml [6].

Oxygen flux, or the amount of O_2 delivered every minute (DO_2) depends on concentration and oxygen saturation of Hb, the cardiac output and the PO_2 (Eq. 3.1). In a person with Hb of 15 g/dL and the cardiac output (Q) of 5 L/min, this amounts to approximately 1000 ml/min.

$$DO_2 = \left\{\left(Hb \times 1.34 \times SaO_2\right) + \left(PaO_2 \times 0.3\right)\right\} \times Q \qquad (3.1)$$

Adequacy of tissue perfusion and oxygenation is crucial for all metabolic processes in the cells and for tissue healing and resisting microbial infection. Although, tissue perfusion could be assessed by capillary refill time, measuring the oxygen tension and pH of the gastrointestinal mucosa provides an objective measurement. Tissue oxygenation can be impaired in smokers and diabetics and when the tissue is hypothermic, while fluid supplementation, hypercapnia, and sympatholytic effect of neuraxial anesthesia may improve tissue perfusion [7]. Perfusion that is inadequate to meet the metabolic demands ushers in anaerobic metabolism, resulting in increased production of lactic acid. Lactic acidosis may be encountered with circulatory shock, severe diabetic ketoacidosis, ethanol overdose, chronic liver disease and use of drugs that inhibit gluconeogenesis and lactate transport (e.g., metformin).

In the tissues, about 250–300 ml of O_2 is consumed every minute, and with an average respiratory quotient of 0.8, approximately 200–240 ml of carbon dioxide (CO_2) is produced during the same period. This CO_2 is transported, by the blood, to the lungs in three forms: (a) dissolved in the plasma (5%), (b) as bicarbonate (85%), and (c) as carbamino compound (10%). The solubility coefficient of CO_2 is 0.06 ml/100 ml/mmHg PCO_2 (0.23 mmol/L/kPa), which is 24 times more than that of O_2. This translates to 3 ml/100 ml of venous blood (P_vCO_2 – 46 mmHg) and 2.5 ml/100 ml in the arterial blood (P_aCO_2 – 40 mmHg). CO_2 from the tissue diffuses into the blood stream and readily enters the RBC. The CO_2 combines with water to form carbonic acid, a reaction that is accelerated by carbonic anhydrase, an enzyme that is present in abundance, in the RBC. Carbonic acid rapidly dissociates into hydrogen ion (H+) and bicarbonate ion (HCO_3^-). This increases the CO_2 carrying capacity of blood. However, for this reaction to continue, the H+ and the HCO_3^- has to be removed from the RBC. The H+ attaches to the imidazole group of the amino acid histidine of the deoxygenated Hb and the HCO_3^- diffuses out of the RBC into the plasma using the 'Band 3' anion transport protein. However, to maintain the electrical neutrality of the RBC each HCO_3^- is exchanged for a chloride ion. This is known as the 'chloride shift' or Hamburger phenomenon [8]. Carbamino compounds are formed by the reversible reaction between CO_2 and the terminal uncharged amino groups (R-NH2) present on the protein molecule, such as hemoglobin (carbaminohemoglobin).

Although, about 200 ml of additional CO_2 is transported every minute from the peripheral tissues to the lung, the dif-

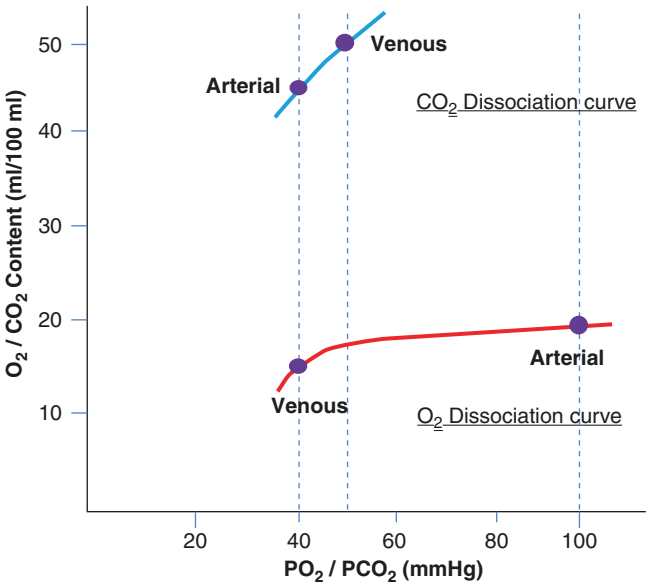


Fig. 3.3 Comparing O_2 and CO_2 content in blood. Although, the difference in content of O_2 and CO_2 between the arterial and venous blood is similar (5 ml/100 ml of blood) the CO_2 dissociation curve is steeper compare to that of O_2 since the difference in partial pressures for O_2 are wider

ference in PCO_2 in the arterial and the venous blood is about 6 mmHg and the pH is not altered. This is possible because deoxygenated Hb is 3.5 times more effective in combining with CO_2 than oxyhemoglobin (Haldane effect) and due to the ability of Hb to buffer H+ and facilitate the formation of HCO_3^-, which is the principal buffer in blood. Although, the difference in content of O_2 and CO_2 between the arterial and venous blood is similar (5 ml/100 ml of blood) the CO_2 dissociation curve is steeper compare to that of O_2 since the difference in partial pressures for O_2 are wider (Fig. 3.3). The term 'CO_2 dissociation curve' is a loose analogy to O_2-Hb dissociation curve, even though hemoglobin is not the major site of CO_2 carriage, unlike O_2 [6].

Transport of Hormones, Nutrients and Metabolic Waste

Hormones secreted by the endocrine system are transported by blood to distant target cells where it exerts its effect. The hormones can be of three chemical types: amines, steroids, or peptides. The amines are synthesized from tyrosine, the steroids from cholesterol and the peptides by transcription by specific mRNA in the endoplasmic reticulum. In the plasma, the hormones are transported bound to the plasma proteins and in its free form. There are specific proteins that bind certain hormones, such as thyroxine-binding globulin and corticosteroid-binding α2-globulin.

Blood is responsible for the transportation of nutrients that are essential for growth and development such as glucose, amino acids, lipids, and vitamins.

Glucose is soluble in water and carried in the plasma. It is absorbed from the intestinal lumen into the intestinal epithelial cells by active transport and into the water portion of the plasma. Glucose entry into the RBC is facilitated by glucose transporters (GLUT-1) which is regulated by the balance of ATP and AMP level within the RBC. A high AMP:ATP ratio promotes glucose uptake into the RBC.

Lipids are transported in plasma as free fatty acid, triglyceride, and cholesteryl ester. Free fatty acid is transported as a physical complex with plasma albumin, while triglycerides and cholesteryl esters, due to their hydrophobic nature, are carried in the core of plasma lipoproteins. Since, lipids are less dense compared to water, the density of lipoproteins decreases as the proportion of lipid to protein increases. Cholesterol and triglycerides produced by the liver are combined with other apoproteins and secreted into the blood as very-low-density lipoproteins (VLDL). Once the triglycerides are removed by a cell the remaining cholesterol-lipoprotein complex becomes low-density (LDL). Cholesterol that is unused by the tissue is returned from these organs to the liver attached to high-density lipoproteins (HDL). This cholesterol is used for bile formation and some of which is excreted, and not deposited into the wall of the blood vessels. Therefore, a high ratio of HDL-cholesterol to total cholesterol suggests protection against atherosclerosis. Six enzymes, together with apolipoprotein cofactors and lipid transfer proteins, facilitate the transport of lipids in blood.

Dipeptides and tripeptides, which are broken down from ingested proteins, are further hydrolyzed into free amino acids within the intestinal epithelial cells. These amino acids are transported via the hepatic portal venous system and taken up by hepatocytes as building blocks for proteins production. Blood is also responsible for the transfer of metabolic end-products that has to be detoxified in the liver before elimination from the body. The amine group of the amino acid is removed by transamination and is accepted by the α-ketoglutarate to form glutamate. Glutamate is transported to the liver, where it gives up the nitrogen as ammonia (NH_3). Ammonia, which is in equilibrium with ammonium (NH_4+), is converted to urea by the urea cycle. [$2NH_3$ (ammonia) + CO_2 + 3 ATP + H_2O → H_2N-CO-NH_2 (urea) + 2 ADP + 4 Pi + AMP].

Urea is the primary nitrogenous waste product of protein metabolism and is carried by blood to the kidneys to be excreted in the urine. Urea, apart from being a carrier of waste nitrogen, also plays a role in the countercurrent exchange system of the nephrons, which allows for reabsorption of water and critical ions from the glomerular filtrate. The process of creating hyperosmotic urine is controlled by the anti-diuretic hormone (ADH) or vasopressin. The other waste products of aerobic metabolism, namely CO_2 and H^+ are also transported by the blood to the lungs and kidneys, respectively.

Hemostasis and Coagulation

The coagulation process is an intricate interaction between the disrupted vessel wall, the aggregated platelets, and the activated clotting factors (Table 3.2). The first response to the disruption of a blood vessel is reflex vasoconstriction of the adjacent blood vessel, which apart from limiting blood loss by slowing blood flow, also activates the platelets. In the plasma, clotting Factors XII, XI, IX, X, VII, prothrombin and fibrinogen exist as inactive precursors that are activated by proteolytic cleavage into serine proteases. Factor VIII is a large protein and made up of two components: the VIIIR-Ag and the VIIIc. The von-Willebrand factor (VIII-WF) forms a part of VIIIR-Ag and is responsible for the platelet adhesion. Fibrinogen is made up of three pairs of polypeptide chains, α, β, and δ. Thrombin separates fibrinopeptide α and β and convert fibrinogen to fibrin monomer. The activated Factor XIII introduces Glu-Lys peptide bonds between the monomers to stabilize the clot.

Table 3.2 Factors that regulate coagulation

	Properties	In vivo half-life (h)
Coagulation factors		
Factor 1 – Fibrinogen	Clot substrate	100–150
Factor II – Prothrombin	Serin-protease zymogen (Vitamin K dependent)	50–120
Factor III – Tissue Factor	Receptor-cofactor	
Factor IV – Calcium	Factor binding to phospholipids	
Factor V – Proaccelerin	Cofactor for X-Prothrombinase complex	12–36
Factor VII – Pro-convertin	Activates IX, X (Vitamin K dependent)	4–6
Factor VIII – Anti-hemophilic A	Complex with IX, activates X with platelet factor 3 & Ca	10–16
Factor IX – Anti-hemophilic B	Complex with VIII, activates X (Vitamin K dependent)	24
Factor X – Stuart-Prower factor	Prothrombinase complex with V(Vitamin K dependent)	36–48
Factor XI – Plasma thromboplastin antecedent	Activates IX	40–80
Factor XII – Hageman factor	Activates XI, VII & prekallikrein	50–70
Factor XIII – Fibrin stabilizing factor	Transglutaminase zymogen	150–300
Prékallilrein (F Fletcher)	It is cleaved by Factor XII to form kallikrein	35
Coagulation inhibitors		
Antithrombin	Inhibits IIa, Xa and other proteases	50–70
Protein C	Inactivates Va & VIIIa (Vitamin K dependent)	6–8
Protein S	Cofactor for Protein C	
Tissue Factor Pathway Inhibitor	Inhibits Tissue factor-Xa-VIIa complex	
Thrombomodulin	Cofactor in thrombin-induced activation of Protein C	

The synchronization of coagulation factors along with coagulation inhibitors and fibrinolysis play a crucial role in the process of clotting and healing

The process of control of the bleeding is also about blood flow through the healed blood vessel. There is an anticoagulant system that exerts a regulatory role over the procoagulant activity to localize the thrombus formation. Antithrombin (AT) is a serine protease inhibitor, which binds and inactivates thrombin, factors IXa, Xa, XIa and XIIa. The enzymatic activity of AT is enhanced in the presence of heparin. Protein C is a serine protease with potent anticoagulant, profibrinolytic and anti-inflammatory properties. It is activated by thrombin and acts by inhibiting activated factors V and VIII, with Protein S and phospholipids acting as cofactors. Thrombomodulin is a transmembrane receptor located on the endothelial cells that binds to thrombin and prevents the formation of the clot over an undamaged endothelium. Tissue factor pathway inhibitor (TFPI) inhibits the TF/VIIa/Xa complex as a mechanism to localize the clotting [9].

Historically, the coagulation cascade was described as activated by tissue factor (extrinsic) or a contact factor (intrinsic). However, a newer, unified concept of 'initiation', 'amplification' and 'propagation', describes this complex process more clearly [10].

Initiation The disruption of the endothelium exposes the circulating coagulation factors to the 'tissue factor', a glycoprotein located in the subendothelial layer. The tissue factor binds to the activated factor VII (proconvertin -VIIa) and this complex activates factors IX and X. Factors IXa and Xa, in the presence of Va, generates a small amount of thrombin (IIa) from prothrombin (II). This thrombin primer 'initiates' the coagulation process by activating the platelets. The surface of the activated platelets forms the template for the coagulation factors to conglomerate.

Amplification The process of 'amplification' essentially increases the production of thrombin that is needed to cleave the fibrinogen to fibrin. The primer amount of thrombin activates non-enzymatic cofactors, factor V and VIII and XI which generates large amounts of factors IXa and Xa.

Propagation On the surface of the activated platelets, factor VIIIa in presence of platelet factor 3 (phospholipids) and calcium ions generate large amounts of Xa. Activated 'Stuart-Prower' factor (Xa) along with Va forms the prothrombinase complex which rapidly cleaves large amount of prothrombin to thrombin (IIa), which in turn breaks down fibrinogen to fibrin. The thrombin also activates factor XIII which transforms the soluble fibrin monomers into a stable fibrin matrix (Fig. 3.4).

Once the organized clot has stopped the bleeding, the enzymatic process of fibrinolysis initiated by plasmin, starts the process of clot lysis and vessel healing. Plasmin is derived by cleavage of the peptide bond of the fibrin-bound plasminogen, by plasminogen activators. The principal plasminogen activators are tissue plasminogen activators (t-PA), synthesized by the endothelial cells, and urokinase-plasminogen activator, synthesized by various cell types including fibroblasts, epithelial cells, and the placenta [10].

Immune System

An effective immune system should be able to differentiate a foreign cell or molecule from self and prepare an effector response to neutralize an invading organism, react to a foreign tissue or eliminate an abnormal cell. The blood and the

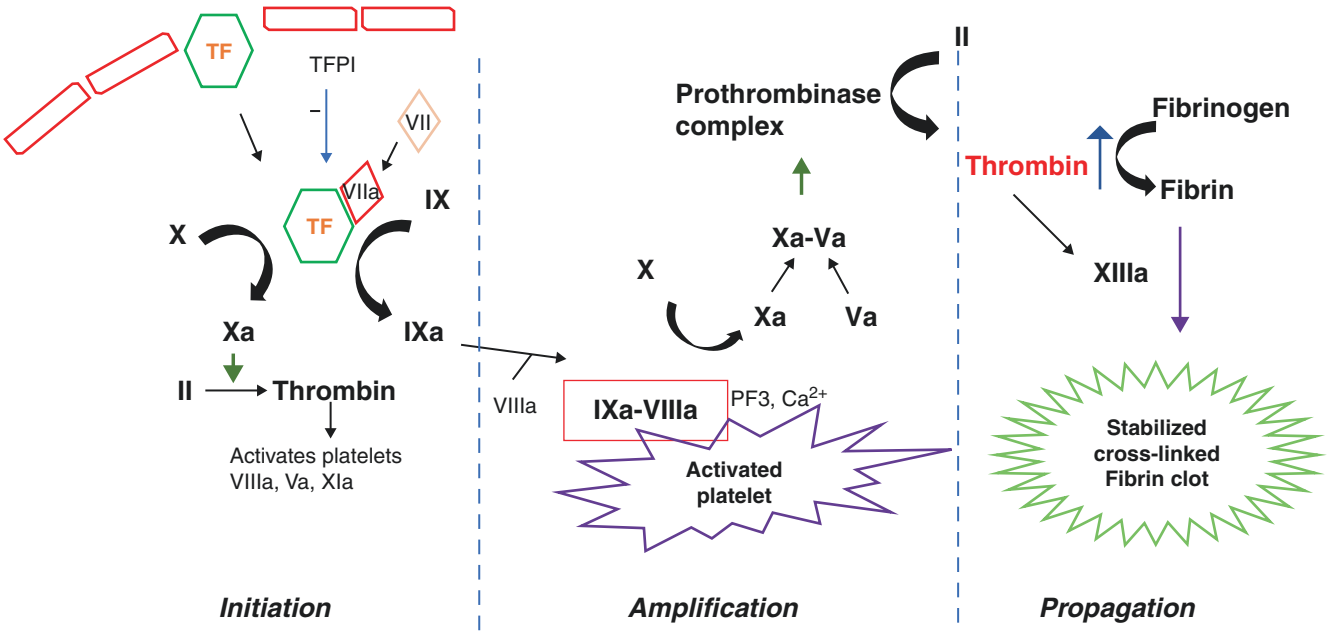


Fig. 3.4 Unified concept of coagulation. The concept of coagulation is better explained as 'initiation', 'amplification' and 'propagation'. TF tissue factor, TFPI tissue factor pathway inhibitor, PF3 platelet factor 3. The roman numerals refer to the clotting factors as alluded to in Table 3.2

lymphoid tissue, along with the bone marrow and the thymus, play an important role in developing and executing this vital defense mechanism. All immune cells, namely lymphocytes and phagocytes, evolve from immature stem cells in the bone marrow, in response to cytokines and other signals. The human immune response can be divided into the 'innate' and the 'adaptive' immunity.

The *innate* defense mechanism not only consists of the anatomical (skin, mucous membrane) and the physiological (temperature, pH, tissue oxygen level) barriers but also a complex system of granulocytes, macrophages and natural killer (NK) cells, collectively known as 'phagocytes', that can recognize a foreign antigen and neutralize it by engulfing the microorganism. The innate immune cells recognize microorganisms by sensing the pathogen associated molecular patterns (PAMP), which are essentially components of microbial metabolism, including proteins, lipids, carbohydrates, and nucleic acids and distinct from self-antigens. The recognition of PAMP is mediated by numerous types of pattern-recognition receptors (PRR) that are expressed on the phagocytes. It has been shown that different PRR react with specific PAMP and can essentially trigger a distinct anti-pathogen response. Hence, contrarily to what was originally thought, the innate immune response may not be completely nonspecific, but rather able to discriminate between self-antigens and a variety of pathogens and may be a prerequisite to the induction of antigen specific adaptive immune response [11].

The granulocytes (neutrophils, eosinophils, basophils, mast cells) contain large cytoplasmic granules of enzymes and microbicidal substances that can be stained by basic dyes. As part of the immune response, the granulocytes migrate to the site of infection and release several molecules, including histamine, cytokines, chemokines, enzymes, and growth factors, which form an integral part of inflammation and the etiology of an allergic reaction. Of the four types, *basophils* are least common (0.5%) and involved in antigen presentation, stimulation, and differentiation of CD4+ T cells; the *eosinophils* (1%) are involved with the pathogenesis of allergic and autoimmune diseases; the *mast cells* are rich in heparin and histamine and involved in varied immunological responses ranging from allergy to immune tolerance; and the *neutrophils* being the most abundant (70%), form the forerunner of the body's cellular immune response. The natural killer (NK) cells are from the lymphocytic lineage and control several types of tumors and microbial infections by limiting their spread and subsequent tissue damage. Recent research highlights that NK cells are also regulatory cells interacting with dendritic cells, macrophages, T cells and endothelial cells, and can thus limit or exacerbate immune responses. NK cell manipulation seems to hold promise in efforts to improve hematopoietic and solid organ transplantation, promote anti-tumor immunotherapy and control inflammatory and autoimmune disorders [12]. Apart from granulocytes and NK cells, there are phagocytic cells in the blood (monocytes), the tissues (macrophage) and the lymphoid system (dendritic cells) that contribute to innate immunity.

The dendritic cells (DC), when activated by the microorganisms or its components, undergo a complex process of

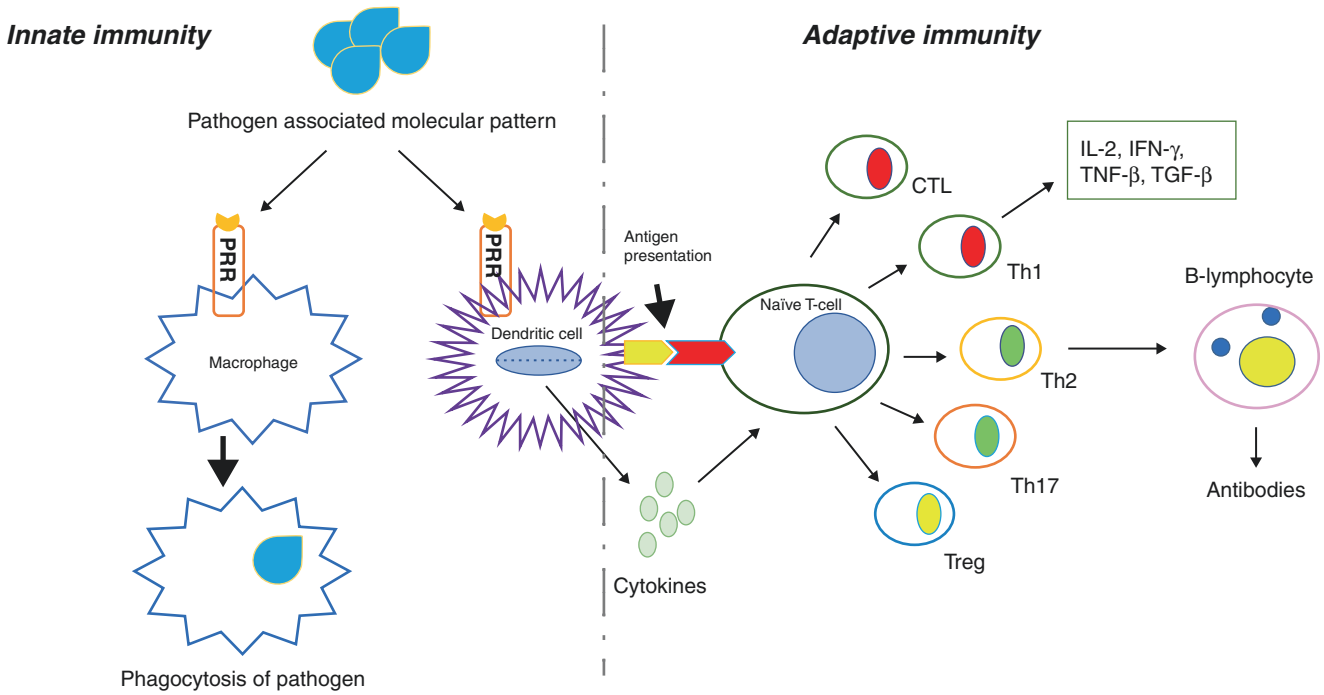


Fig. 3.5 The innate and adaptive immunity. The innate immunity can discriminate between self-antigens and a variety of foreign proteins and is a prerequisite to the induction of antigen specific adaptive immune response. PRR pattern-recognition receptors, IL interleukin, IFN interferon, TNF tumor necrosis factor, TGF transforming growth factor, CTL cytolytic T lymphocyte, Th helper T cells, Treg regulator T cells

morphological and functional modification. These mature DC enters the draining lymphatic vessels and migrate to the T-cell zones of the draining lymph nodes, where they present the 'foreign' antigen to the T lymphocytes. Depending on their maturation profile, DC will stimulate the proliferation of distinct T cells or maturation of B lymphocytes (Fig. 3.5). Therefore, the innate immunity is not only responsible for the infection-induced nonspecific inflammatory response but also setting up the specific adaptive immunity to the invading pathogens [11]. This adaptive immunity also develops immunological memory, allowing the host to rapidly respond when exposed to the same pathogen later in time.

B lymphocytes develop in the bone marrow and the immunoglobulins on their surface is crucial for antigen recognition and binding. Once activated, the B lymphocytes mature into antibody-secreting plasma cells. The antibody released from the B cell binds to the antigen to form an immune complex, which along with the complement cause lysis of the microorganism. Immunoglobulins (Ig) are proteins that consists of two identical light chains and two heavy chains joined by disulfide bonds. The unique heavy chain sequence determines five isotypes of immunoglobulins: IgG, IgM, IgA, IgE, and IgD.

IgG works efficiently to coat microbes, enhancing their uptake by other cells in the immune system; IgM is highly effective at killing bacteria; IgA are present in the secretions of the mucous linings such as tears, saliva, secretions of the respiratory and the digestive tract; IgE, whose natural function is probably to protect against parasitic infections, is also responsible for the symptoms of allergy; and IgD remains attached to B cells and plays a key role in initiating early B-cell response.

The T lymphocytes leave the bone marrow and mature in the thymus and can only recognize the antigen that is bound to the major histocompatibility complex (MHC) molecule. Although discovered during studies on transplanted tissue compatibility, MHC molecules or the *human lymphocyte antigen* (HLA) are cell surface proteins essential to discriminate an alien tissue or molecule from one's own. T lymphocytes are of two types, the cytotoxic T lymphocytes (TC) and the T helper lymphocytes (TH). TC expresses CD8 cell marker which recognizes foreign antigen on the HLA I, which is expressed on all cells, except the RBC, and induces apoptosis of the cell displaying the antigen. TH expresses the CD4 membrane glycoprotein which recognizes the antigen displayed on HLA II that are expressed on antigen presenting cells, like macrophages, dendritic cells, and the B lymphocytes.

The complement system is a group of serum proteins that have an important role in antigen clearance. These are activated either by specific immunoglobulin molecules (classic pathway) or a variety of microorganisms and immune complexes (alternative pathway). The complement proteins amplify antigen-antibody reactions, attract phagocytes to the site of infection, augment phagocytosis, and activate B lymphocytes. These proteins cause blood vessels to become

dilated and leaky, causing redness, warmth, swelling, pain, and loss of function that characterizes an inflammatory response. Cytokines are chemical messengers that establish communication between the components of the immune system. These are a diverse group of proteins which include interleukins, interferons, and growth factors.

Acid–Base Regulation

Although the human body is an acid producing organism, the pH of the blood is maintained relatively stable at a slightly alkaline level of 7.35–7.45. An acid is a compound that releases a H+ (proton) and a base is one that can accept it. pH is the negative logarithm of H+ concentration in moles/L. The intracellular pH is 6.8 ([H+] = 160 nmol/L), while the pH of the extracellular fluid is maintained at 7.35–7.45 ([H+] = 35–45 nmol/L). This four-fold concentration gradient of [H^+] is counterbalanced by the intracellular potential of −70 mV.

A buffer is a substance that minimizes the change in pH of a solution when a strong acid or alkali is added to that solution. It is a mixture of a weak acid with its conjugate base. The buffer is most effective when the pH of the milieu is close to its pKa, which is the pH at which it is 50% ionized. The relationship between the pH and the pKa is demonstrated by the Henderson-Hasselbalch equation.

$$\left(\textbf{Henderson}-\textbf{Hasselbalch}\right):\ \mathrm{pH}=\mathrm{pKa}+\log_{10}\frac{[\mathrm{base}]}{[\mathrm{acid}]} \tag{3.2}$$

The extracellular buffer systems available in the blood are (a) the bicarbonate system (H_2CO_3/HCO_3^-), (b) the hemoglobin system (HHb/Hb^- & $HHbO_2/HbO_2^-$), (c) the protein (histidine) system, and d) the phosphate ($H_2PO_4^-$/ HPO_4^{2-}) system.

The bicarbonate system has a pKa of 6.1 and using the Henderson-Hasselbalch equation, it can be derived that the pH of 7.4 is dependent on maintaining the ratio of HCO_3^- to CO_2 of about 20 [6].

$$\mathrm{pH}=6.1+\log_{10}\frac{\left[\mathrm{HCO_3^-}\right]}{\mathrm{S}\times\mathrm{PCO_2}}$$

(S is the solubility coefficient of CO_2: S = 0.03 when PCO_2 is expressed in mmHg)

The components of the bicarbonate system, namely CO_2 and HCO_3 are in equilibrium and the conversion of CO_2 to H_2CO_3 and then to HCO_3^- is catalyzed by carbonic anhydrase. Carbonic anhydrase is present in high concentration in the erythrocytes and the renal tubular cells and both these cells can remove H+, thereby favoring the production of bicarbonate.

The versatility of the bicarbonate system is that the level of CO_2 and the HCO_3^- can be regulated by altering the ventilation and the renal excretion, respectively. The HCO_3^- is not only responsible for 60% of total buffering capacity of the blood, its presence is needed for efficient buffering by hemoglobin, which constitutes most of the remaining buffering capacity and it is also necessary for H+ excretion by the kidneys.

Hemoglobin is an important buffer, but it needs the bicarbonate buffer system to be effective. The increase in CO_2 in the venous blood leads to increase production of HCO_3^- which tends to maintain the HCO_3^-/CO_2 ratio, thus minimizing the change in pH. A molecule of deoxygenated hemoglobin can accept 0.7 mmol of H^+.

The phosphate system ($H_2PO_4^-/HPO_4^{2-}$) has a pKa of 6.8 and therefore theoretically better than the bicarbonate system, but its concentration in the plasma is only 3.1 mg/dL (1 mmol/L). It does have a significant role as a buffer within the cell and in the urine.

Plasma proteins can buffer H^+ but are only about 15% as effective as hemoglobin.

Thermoregulation

Humans being homeothermic, maintain their core body temperature between 36.5–37.3 °C. This relatively narrow range is optimal for enzyme function and chemical reactions within the cells. The human body may be considered as two compartments: the '*core*' consists of the trunk organs and the brain while the limbs, skin, and the subcutaneous tissue make up the '*periphery*'. The blood functions as the conduit for transfer of heat energy between the two compartments. In health, the core temperature is maintained reasonably constant, while the skin temperature fluctuates widely mirroring the ambient temperature. The core temperature is regulated and stabilized primarily by the anterior hypothalamic nucleus and the adjacent preoptic area regions of the hypothalamus [13]. The autonomic thermoregulatory response to cold is vasoconstriction and shivering, while the response to warmth is sweating and vasodilatation of arteriovenous shunts located in the dermis.

Regulation of Body Fluids

The volume and the composition of the intracellular (ICF) and the extracellular (ECF) fluid compartments is determined by the movement of water, electrolytes, and plasma proteins between the two. Osmosis is the movement of

water across a semipermeable membrane when there is an inequality in the concentration of non-diffusible solutes across it. The osmotic pressure is determined by the concentration gradient of osmotically active particles across the membrane and is proportional to the number of molecules or ions and not their molecular mass or charge. The osmolarity of a solution is the number of osmotically active particles per liter of solution, while the osmolality is the measure of such particles per kilogram of solvent. Unlike osmolarity, Osmolality is unaffected by the temperature or pressure. When considering dilute body fluids, which are primarily water, the difference between osmolarity and osmolality is negligible and used interchangeably. Since all the solutes in the plasma are measured in liters, osmolarity is commonly used.

Osmotically active solutes reduce the freezing point of the solute. The freezing point of plasma is about 0.54 °C, which corresponds to an osmolarity of 290 mOsm/L. Most of this is contributed by Na+ and the accompanying anion. Proteins contribute <1 mOsm/L. The significant non-ionic contributor of plasma osmolarity are glucose and urea or the blood urea nitrogen (BUN).

$$\text{Osmolarity}\left(\text{mOsm/L}\right) = 2\times\left[\text{Na+}\right] + \left(\text{glucose}\right) + \left(\text{urea}\right)\left[\text{glucose \& urea in mmol/L}\right]$$

$$\text{Osmolarity}\left(\text{mOsm/L}\right) = 2\times\left[\text{Na+}\right] + 0.055\left(\text{glucose}\right) + 0.36\left(\text{BUN}\right)\left[\text{glucose \& BUN in mg/dL}\right]$$

The osmolarity of the ICF and ECF are similar as water is freely permeable between the two and therefore, the osmolarity of plasma is a guide to intracellular osmolarity.

In 1896, Ernest Starling suggested a hypothesis that the movement of water across the endothelium is dependent on the balance between the hydrostatic pressure gradient and the oncotic pressure gradient across the capillary [14].

$$\left(\textbf{Starling hypothesis}\right): \text{Jv} = \text{Lp.S}\left(\text{Pc} - \text{Pi}\right) - \sigma\left(\pi c - \pi i\right) \tag{3.3}$$

Where:

- Jv – trans endothelial solvent filtration volume per second
- Lp – hydraulic conductivity of the membrane
- S – surface area for filtration
- Pc – capillary hydrostatic pressure
- Pi – interstitial hydrostatic pressure
- πc – plasma protein oncotic pressure
- πi – interstitial oncotic pressure
- σ – Staverman's reflection coefficient, a measure of the leakiness to plasma proteins

The net driving pressure is outward at the arteriolar end and inward at the venous end of the capillary, because of the variation in hydrostatic pressure. This suggestion was based on Poiseuille's work (1799–1869) and the thinking that small molecules and electrolytes are freely permeable across the capillary endothelium, while the plasma proteins remain confined to the intravascular compartment. Albumin, the principal plasma protein exerts a colloid oncotic pressure of about 25 mmHg, which opposes the movement of water out of the blood vessel. However, over the last 50 years a better understanding of the microcirculation has shown the existence of the endothelial glycocalyx, a pre-capillary sphincter and a capillary wall that is porous to plasma protein [15]. This suggests that the Starling's hypothesis may not be sufficient to completely explain the physiology of the microcirculation. The blood and the lymphatic system are crucial in maintaining the balance of plasma and the interstitial fluid.

Conclusion

Blood is a perfect amalgamation of cells, proteins, electrolytes, and water that delivers nutrients and oxygen to the cells, carries metabolites and CO_2 to be excreted, protects the body against microbial infection and bleeding, and helps in regulating the acid-base balance, body temperature and the composition of the body fluids. The fluidity of blood is crucial for its ability to perform its various functions and sustaining human life.

Key Points

- Blood is a connective tissue made up of a myriad of cells in a fluidic extracellular matrix.
- The cells in the blood differentiate from either the common myeloid progenitor cell or the common lymphoid progenitor cell, which is derived from the pluripotent hematopoietic stem cell within the bone marrow.
- The main functions of blood can be classified broadly into transport, defense, and homeostasis.
- The hemoglobin molecule is adapted to carry oxygen and facilitates the transport of CO_2.

- The coagulation process is an intricate interaction between the disrupted vessel wall, the aggregated platelets, and the activated clotting factors.
- The innate immunity can discriminate between self-antigens and a variety of foreign protein and is a prerequisite to the induction of antigen specific adaptive immune response.
- Blood plays a crucial role in thermoregulation and maintaining the composition of body fluids.

References

1. Yan H, Hale J, Jaffray J, Li J, Wang Y, Huang Y, An X, Hillyer C, Wang N, Kinet S, Taylor N, Mohandas N, Narla A, Blanc L. Developmental differences between neonatal and adult human erythropoiesis. Am J Hematol. 2018;93(4):494–503. https://doi.org/10.1002/ajh.25015. Epub 2018 Jan 9. PMID: 29274096; PMCID: PMC5842122.
2. Nandakumar SK, Ulirsch JC, Sankaran VG. Advances in understanding erythropoiesis: evolving perspectives. Br J Haematol. 2016;173(2):206–18. https://doi.org/10.1111/bjh.13938. Epub 2016 Feb 5. PMID: 26846448; PMCID: PMC4833665.
3. Romano O, Petiti L, Felix T, Meneghini V, Portafax M, Antoniani C, Amendola M, Bicciato S, Peano C, Miccio A. GATA factor-mediated gene regulation in human erythropoiesis. iScience. 2020;23(4):101018. https://doi.org/10.1016/j.isci.2020.101018. Epub 2020 Mar 30. PMID: 32283524; PMCID: PMC7155206.
4. Ponka P, Koury MJ, Sheftel AD. Erythropoiesis, hemoglobin synthesis, and erythroid mitochondrial iron homeostasis. In Ferreira GC, Kadish KM, Smith KM, Guilard A (Eds) The handbook of porphyrin science. World Scientific Publishers, Singapore. https://doi.org/10.1142/9789814407755_0011.
5. Brown KA. Erythrocyte metabolism and enzyme defects. Lab Med. 1996;27:329–33. https://doi.org/10.1093/labmed/27.5.329. Assessed 20 Feb 2021.
6. West JB, Luks AM. Gas transport by the blood. In: West's respiratory physiology: the essentials. 10th ed. Philadelphia: Wolters Kluwer; 2016. p. 87.
7. Kabon B, Nagele A, Reddy D, Eagon C, Fleshman JW, Sessler DI, Kurz A. Obesity decreases perioperative tissue oxygenation. Anesthesiology. 2004;100:274–80.
8. Westen EA, Prange HD. A reexamination of the mechanisms underlying the arteriovenous chloride shift. Physiol Biochem Zool. 2003;76:603–14.
9. Roberts HR, Monroe DM, Escobar MA. Current concepts of hemostasis: implications for therapy. Anesthesiology. 2004;100:722–30.
10. Lasne D, Jude B, Susen S. From normal to pathological hemostasis. Can J Anesth. 2006;53:S2–11.
11. Pathogen strategies to evade innate immune response: a signaling point of view. https://www.researchgate.net/publication/256257651. Accessed 22 Dec 2020.
12. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008;9:503–10.
13. Charkoudian N. Skin blood flow in adult human thermoregulation: how it works, when it does not, and why. Mayo Clin Proc. 2003;78:603–12.
14. Starling EH. Factors Involved in the Causation of Dropsy. Lancet. 1886;2:1266–70.
15. Ghanem AN, Ghanem SA. Volumetric overload shocks: why is starling's law for capillary interstitial fluid transfer wrong? The hydrodynamics of a porous orifice tube as alternative. Surg Sci. 2016;7:245–9.

Hemoglobin: Physiology and Hemoglobinopathy

4

Soojie Yu

Abbreviations

2,3-DPG	2,3 Diphosphoglycerate
CO	Carbon monoxide
CO_2	Carbon dioxide
fL	Femtoliters
g/dL	Grams/deciliter
Hb	Hemoglobin
HbA	Hemoglobin A
HbA2	Hemoglobin A2
HbE	Hemoglobin E
HbF	Hemoglobin F
HbH	Hemoglobin H
HbS	Hemoglobin S
NO	Nitric oxide
O_2	Oxygen
pg	Picograms
R state	Relaxed
RBCs	Red blood cells
SCD	Sickle cell disease
T state	Tense
TI	Beta-thalassemia intermedia
TM	Beta-thalassemia major

Introduction

Hemoglobin has multiple functions that include transporting oxygen from the lungs to the tissues, carrying carbon dioxide from tissues to the lungs, buffering of hydrogen ions and metabolizing nitric oxide. These functions can be altered in individuals who have hemoglobin disorders. Hemoglobin disorders include hemoglobinopathies, which are structural changes in the globin proteins of hemoglobin, and thalassemia, which are mutations in globin expression. Depending on the amount of mutated hemoglobin present individuals can have a variety of severity of symptoms.

Hemoglobin and Oxygen Binding Regulation

Hemoglobin (Hb) is produced in erythroid cells in the bone marrow of long bones and flat bones. It is a tetrameric protein composed of four globin chains. Each chain contains a heme molecule which consist of a protoporphyrin ring and a central iron ion in the ferrous state (Fe^{2+}) [1, 2]. In adults, there are two major tetramers, Hemoglobin A (HbA) which have two α and two β globin polypeptides, and Hemoglobin A2 (HbA2), which have two α and two δ globin polypeptides. HbA is the most abundant form (>90%) of adult Hb [3]. In fetuses, the majority of hemoglobin is hemoglobin F (HbF), which contains two α and two γ polypeptides [2]. HbF level declines approximately 6 months after birth [2]. The globin chains are encoded by the α and β gene clusters on chromosome 16 and 11, respectively [4]. The α-like genes include an embryonic gene (ζ) and two adult genes (α1 and α2) while the β-like genes include an embryonic gene (ε), fetal gene (γ), and adult genes (β and δ) [4]. Hb is an allosteric protein that exists in two forms a tense (T) state and a relaxed (R) state [2]. When heme binds to oxygen (O_2), the ferrous ion is pulled closer to the protoporphyrin ring flattening the ring and changing the shape [2]. In the globin chain, the heme molecule is located in a crevice on the side. In the T state, the crevices are small making it difficult for oxygen to bind to heme [2]. When one heme molecule binds to oxygen, it causes the structural change relaxes the molecule and opens up the crevices on adjacent globin therefore increasing their affinity to oxygen [2]. When all four hemes are bound to oxygen, Hb is then in its relaxed (R) state [2]. Hb only binds oxygen when iron heme is in its ferrous (2+) form [5]. If iron heme is oxidized to its ferric form (3+), it is called methemoglobin and it no longer binds to oxygen [2, 5]. Hb can have a harmful role under stress or pathological condi-

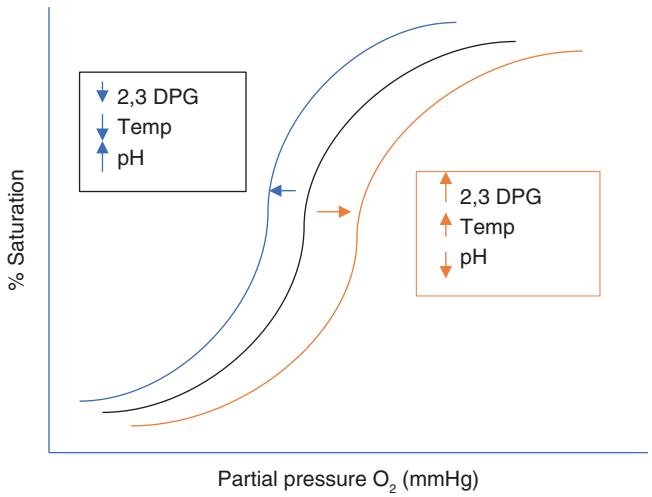
S. Yu (✉)
Department of Anesthesiology, Mayo Clinic Arizona, Scottsdale, AZ, USA
e-mail: yu.soojie@mayo.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_4

tions. In acidic environment, oxygen attached to Hb can accept an electron from the ferrous ion to form a superoxide and a ferric ion or a methemoglobin [6]. Within the RBC, antioxidants help reduce the ferric ion to the ferrous state but outside the RBC, auto-oxidation is high [6].

Oxygen Binding

Oxygen saturation, which is the percentage of oxygen bound to Hb, is different from partial pressure of oxygen in the blood, which is the amount of oxygen dissolved in the blood. Approximately 98% of oxygen in the blood is bound to Hb while 2% is dissolved in the plasma [7]. The oxyhemoglobin dissociation curve helps describe the relationship between the concepts [2, 8]. The oxyhemoglobin dissociation curve has a sigmoid shape due to the positive cooperativity and change in affinity after subsequent binding of oxygen to other heme molecules [2]. Initially, when the first oxygen molecule binds to deoxyhemoglobin, the affinity is low, and the partial pressure of oxygen is low. The curve at that point is flat. After one oxygen molecule binds, it becomes easier for each subsequent oxygen molecules to bind; therefore, increasing the slope of the graph. As the partial pressure of oxygen increases and most of the oxygen binding sites are occupied, the curve flattens again. In physiologic conditions, the venous oxygen saturation is around 75% or above which implies normally the final oxygen binding site is occupied or unoccupied in hemoglobin making it very efficient [2]. P50 is another important point on the oxyhemoglobin dissociation curve. P50 is the partial pressure of oxygen in blood when hemoglobin is 50% saturated. Changes in the P50 value helps describe if the curve is shifted towards the right or left which indicates if the affinity of oxygen is decreased or increased, respectively [2].



Other factors that affect the affinity of Hb to oxygen are the pH, partial pressure of carbon dioxide (CO_2), temperature and 2,3 diphosphoglycerate (2,3-DPG) [2]. A decrease in pH causes the oxyhemoglobin dissociation curve to shift to the right due to increases in hydrogen ions stabilize hemoglobin in its deoxygenated form [7]. As pH decreases and CO_2 increases, hemoglobin affinity for oxygen decreases. CO_2 affects the dissociation curve in two ways. The majority of CO_2 is converted into carbonic acid within erythrocytes [7]. Carbonic acid dissociates into hydrogen ions and bicarbonate therefore increasing hydrogen ions which decreases the affinity of oxygen to heme [7]. The other method is by binding to heme and forming carbaminohemoglobin which stabilizes the tense deoxygenated state [7].

2,3-DPG

2,3-DPG is an intermediate product of glycolysis and is produced in erythrocytes. It is an anionic phosphate that binds between the beta globin chains of deoxyhemoglobin altering the structure and promoting the release of oxygen from hemoglobin [2]. 2,3-DPG is the principal regulator of oxygen affinity in red blood cells (RBCs). Its binding separates the two β chains which causes favoring of the deoxygenated form and decreasing the affinity of oxygen [4]. When concentrations of 2,3-DPG are high, the dissociation curve is shifted towards the right and when concentrations are low, the curve is shifted towards the left [2]. This helps to explain why oxygen affinity is reduced in anemic patients because there is a higher concentration of intracellular 2,3-DPG which helps increase oxygen delivery to the tissues [4].

Gas exchange in the capillaries occur through the balance of the Bohr and Haldane effects. In the lungs, the partial pressure of oxygen is high therefore deoxygenated hemoglobin is exposed to high amounts of oxygen facilitating oxygen binding [7, 9]. As blood leaves the lungs, the decrease in partial pressure of oxygen in the tissues and increase in CO_2 helps decrease affinity of oxygen and helps release of oxygen from Hb. As the partial pressure of O_2 decreases along the systemic capillary, hemoglobin deoxygenates and the molecule changes toward its tense form. In the tense structural form, CO_2 has increase affinity to hemoglobin Also, the increase in partial pressure of CO_2 in tissues leads to an increase in hydrogen ions [9]. Increase in hydrogen ion concentration promotes the deoxygenated conformation of hemoglobin which shifts the dissociation curve to the right leading to decreased oxygen affinity [4, 7]. When demands exceed the oxygen available, tissue hypoxia can occur which leads to increase lactic acid in tissues. Decreasing oxygen affinity allows for rapid release of oxygen to hypoxic tissues. Decreased oxygen in blood increases production of erythro-

poietin which stimulates the growth and production of red blood cells.

As normally humans are isothermic, temperature has little effect on oxygen affinity to hemoglobin. During exercise, the higher temperatures generated shift the curve to the right and increase oxygen unloading [2]. Also with exercise, the increase in hydrogen ion concentration also shifts the curve to the right favoring oxygen release to the tissues [4].

The binding affinity of heme from highest to lowest is nitric oxide (NO), carbon monoxide (CO) then oxygen [6]. Hemoglobin has 200–300 times higher affinity to carbon monoxide compare to oxygen [9]. When CO binds to Hb, carboxyhemoglobin is formed. Oxygen is prevented from binding due to competition for the binding sites on heme and CO bind nearly irreversibly to heme [9]. When one CO molecule binds to hemoglobin, it increases affinity of the other binding sites for oxygen shifting the oxyhemoglobin dissociation curve to the left [9]. Due to increase affinity for oxygen, release of oxygen to peripheral tissues can be decrease causing severe tissue hypoxia [7].

Nitric oxide (NO) is a signaling molecule for multiple diverse actions. It is produced by vascular endothelial cells and diffuses to smooth muscle cells. In smooth muscle cells, it activates guanylate cyclase which converts guanosine triphosphate into cyclic guanosine monophosphate [5]. NO has a high affinity for the ferrous heme therefore sequestering it due to a slow dissociation rate [5]. NO deoxygenation through reaction with oxyHb destroys NO activity. If NO binds to Hb in the T state, a Hb-NO complex forms with little vasodilatory activity [2]. When NO binds to Hb in the R state, the ferrous ion is oxidized creating methemoglobin and NO is converted to nitrate [2] NO binding to ferric heme does not hinder NO signaling. NO binding to metHb is high but also dissociation rate is fast. Cell-free Hb reacts to NO 1000 times faster than red cell encapsulated Hb [5]. When Hb is in the ferrous state, then it limits the NO signaling preventing it from reaching the smooth muscle. When Hb is reduced to its ferric state, then NO diffusion to smooth muscle is not prevented.

Hemoglobinopathies

Hemoglobinopathies occur when there is a mutation affecting the hemoglobin chain structure while thalassemia syndromes (Tables 4.1 and 4.2) are created when there is a mutation in hemoglobin chain production and expression [2]. Hemoglobin disorders have a high prevalence due to natural selection. Hemoglobin disorders provide relative protection against certain infections like malaria [10]. The improved survival of heterozygotes offset the reduce reproductive success of homozygotes. As there is overlap in geography where hemoglobin disorders are prevalent, co-inheritance of more than one hemoglobin abnormality is common which causes a wide range of clinical phenotypes [4].

Table 4.1 Forms of alpha thalassemia

Gene mutation inheritance	Variant	Phenotype	Treatment
a-/aa	Alpha thalassemia silent carrier	Asymptomatic	
--/aa	Alpha thalassemia trait	Asymptomatic	
--/a-	Hemoglobin H disease	Variable expression, hemolytic anemia, splenomegaly	Episodic transfusions, iron chelation
--/--	Hemoglobin Bart's	Severe anemia, hydrops in affected fetuses	Prenatal screening and counseling, intrauterine transfusions

Table 4.2 Forms of beta-thalassemia

Genotype	Variant	Phenotype	Treatment
B^0/B B^+/B	Beta thalassemia minor/trait	Asymptomatic, mild anemia, MCV and MCH decreased,	
B^+/B^+ B^+/B^0	Beta thalassemia intermedia	Variable degrees of severity, moderate hemolytic anemia, MCV and MCH decreased	Episodic transfusions
B^0/B^0	Beta thalassemia major	Transfusion dependent hemolytic anemia, MCV and MCH decreased, failure to thrive, feeding problems, splenomegaly	Periodic and life-long blood transfusions Hematopoietic stem cell transplant

Thalassemia Syndromes

Thalassemia syndromes are a group of disorders caused by decreased expression of one or more of the globin chain subunits [11]. As a group, they are the most common single genetic disorder [12]. A decrease in expression of one of the globin chains cause increase expression of the unaffected globin chain which create abnormal RBC maturation. Alpha-thalassemia is an inherited, autosomal recessive disorder [1]. Majority of alpha-thalassemia disorders are caused by deletions in the gene and minorly by point mutations affecting the expression of one or more of the alpha globin genes [1]. As the alpha genes are duplicated on each chromosome, complete absence of alpha globin chain only occurs if all four of the genes are deleted. With a mutation affecting the alpha gene on one chromosome, an individual is said to have

"silent" alpha-thalassemia [1]. When two genes are involved, it's alpha-thalassemia trait [1]. With compound homozygotes or heterozygotes, three of four alpha globin genes lose their function due to deletions or mutations creating hemoglobin H (HbH) [1, 13]. Due to the instability and high oxygen affinity, HbH produces intracellular precipitates that decreases the integrity of the RBC membrane therefore leading to ineffective RBC production and early cell death [13]. The most severe form of alpha thalassemia is with no expression of alpha globin genes and is called Hb Bart's Hydrops Fetalis Syndrome [1]. Hemoglobin Bart's have an extremely high oxygen affinity therefore it is ineffective for oxygen transportation [4].

Clinical presentations of alpha-thalassemia can vary from normal to severe anemia with Hb levels ranging 7.5–15.5 g/dL, reduced mean corpuscular volume <79 fL, mean corpuscular hemoglobin <27 pg, a normal to increase red blood cell count, and normal to slightly decreased HbA2 percentage [13–15]. Silent alpha thalassemia or silent alpha thalassemia trait is clinically asymptomatic and identified by chance after routine screening or antenatal screening [1, 16]. HbH disease occurs from compound heterozygosity for two different mutations or from homozygotes mutations [13]. The alpha globin gene is expressed below 30% of normal. HbH disease individuals present with hemolytic anemia, splenomegaly, and other complications [13, 15]. HbH has become the most challenging hemoglobinopathy due to rising genotype patterns and varying phenotypic presentation [13, 15]. The variation in presentation is influenced by the degree of alpha globin deficiency, environmental and genetic factors [13]. When alpha globin chain synthesis falls below 70% of normal in the fetal period, the increase in gamma globin chains form Hb Bart's which can be detected and quantified during newborn screening [14–16]. Hb Bart's Hydrops Fetalis Syndrome clinical features include intra-uterine anemia, hepatosplenomegaly, cardiovascular deformities, skeletal deformities, retardation of brain growth and edema [1]. There is a high risk of intra-uterine death and obstetric complications [1].

β-Thalassemias are heterogenous autosomal recessive disorders that are mostly due to point mutations on the beta-globin gene on chromosome 11 causing abnormal production or expression [10, 16]. In individual ethnic or geographical groups, a majority of beta thalassemia is due to four or five specific mutations [4]. There are three main forms of beta-thalassemia: beta-thalassemia major or Cooley's anemia and Mediterranean anemia; beta-thalassemia intermedia; and thalassemia minor. The main forms are variants of homozygous, compound, or heterozygous mutations or deletion of the beta globin gene [10, 16].

Individuals with beta-thalassemia major (TM) or transfusion dependent thalassemia have severe impairment of beta globin chain production causing accumulation of alpha globin chains and precipitation in precursor RBCs [4]. TM individuals often show symptoms including failure to thrive, progressive paleness, feeding problems, diarrhea, and progressive enlargement of the abdomen due to splenomegaly between the 6–24 months of age [10, 14, 16, 17]. They have microcytic anemia, hemoglobin levels less than 7, mean corpuscular volume >50 < 70 fL, and mean corpuscular hemoglobin >12 < 20 pg [10, 14, 16, 17]. TM individuals require regular blood transfusions to survive. With regular transfusions, abnormal RBC production is prohibited but without compliance with chelation therapy, individuals can develop complications with iron overload [10]. If unable to have regular transfusions, individuals will have growth retardation, poor musculature, hepatosplenomegaly, and skeletal changes from expansion of the bone marrow [10]. Also, without regular transfusions, most die from high output heart failure [10].

β-thalassemia intermedia (TI) or non-transfusion dependent thalassemia individuals present later than TM, have milder anemia, and either do not require or require less transfusions [10, 16, 17]. They have hemoglobin levels around 7 and 10 g/dL, mean corpuscular volume >50 < 80 fL, and mean corpuscular hemoglobin >16 < 24 pg [10]. If persons with TI have symptoms, they present with pallor, mild jaundice, liver and spleen enlargement, tendency to develop osteopenia, and thrombotic complications [10]. Individuals can have high-output cardiac output and pulmonary hypertension [10]. Persons can have iron overload from increased intestinal absorption due to increased production of abnormal RBC [10].

Hemoglobin E (HbE) most common form of thalassemia in southeast Asia and is caused by replacement of a glutamic acid with lysine in the beta globin chain [11]. Homozygote HbE individuals have mild globin-chain imbalance and have similar phenotype to beta thalassemia heterozygotes [18]. HbE can be co-inherited with alpha and beta thalassemia which can produce a variety of clinical syndromes [18].

Alpha thalassemia is predominantly found in tropical and sub-tropical areas. The high carrier rate is believed to be due to the carriers have better protection against malaria falciparum [1]. The severe forms are predominantly found in South East Asia, Mediterranean area and the Middle East [1]. Beta Thalassemia is located throughout tropical and North Africa, the Mediterranean, the Middle East, and south and east Asia [4].

Sickle Cell Disease

Structural variants causing hemoglobinopathies can be expressed as a clinical disease like sickle cell anemia and other sickling diseases, hemolysis due to unstable structures, hemoglobin with altered oxygen affinity, and hemoglobin unable to maintain iron in ferrous state [16]. For sickle cell

disease (SCD), an amino acid substitution in the beta globin chain causes polymerization of mutant hemoglobin S (HbS) [19]. Sickle cell disease is an autosomal recessive disorder that encompasses all disorders where there is a mutation in the beta globin gene that results in the same clinical syndrome [20]. Sickle cell anemia is the most common form and is the first human disorder to be understood at the molecular level [4, 20]. It is caused by homozygous of beta-S allele on chromosome 11 [20]. The change in the nucleotide replaces a hydrophilic glutamic acid with a hydrophobic valine residue [19]. The change causes a mutated hemoglobin tetramer. Deoxygenated Hb which contain two mutant beta chain subunits causes exposure of the hydrophobic residue [3]. The hydrophobic residues bind to each other initiating nucleation of a HbS polymer [20]. The polymers increase cellular rigidity and distort the RBC membrane leading to sickling, stress and premature hemolysis [20]. Polymerization occurs faster with higher concentrations of HbS and occurs slower with higher concentration of HbF [19]. HbF is excluded from HbS polymerization therefore interrupting the polymerization of deoxygenated HbS [20].

HbS polymerization depends on HbS concentration, partial pressure of oxygen, temperature, pH, 2,3-DPG concentration and presence of different Hb molecules [3]. HbS has decreased affinity for oxygen compared with HbA. The decreased affinity exacerbates HbS polymerization which further reduces HbS affinity to oxygen [3, 20]. 2,3 DPG also decreases HbS affinity for oxygen and levels of 2,3 DPG is elevated in sickle erythrocytes [3, 19, 20]. Repeated episodes of RBC sickling and unsickling in conditions of low partial pressure of oxygen to high partial pressure of oxygen causes alterations in membrane structure and function and abnormal calcium compartmentalization [3, 19, 20]. Eventually, RBC sickling is permanent and can no longer revert to natural form.

Geographical distribution of the sickle cell mutation is dependent on the endemicity of malaria and population movements [3]. HbS provides protection against severe P. falciparum malaria therefore the highest frequency of sickle cell allele seen in sub-Saharan Africa, parts of the Mediterranean, the Middle East and India [3]. Variability of the clinical symptoms is partly due to genetic modifiers that affect HbF level and co-inheritance of alpha thalassemia. Expression of HbF ranging from 10–25% of total Hb reduces the clinical severity of sickle cell anemia [3, 19, 20].

Individuals with SCD have propensity for blood vessel occlusion which leads to ischemia and acute systemic painful vaso-occlusive crisis. Vaso-occlusive crisis is frequently initiated by an inflammatory or environmental stimulus including infection, hypoxia, dehydration, or acidosis. Blood vessel occlusion occurs due to the impaired RBC polymerization, increased interaction between RBC's, inflammatory cells, and endothelium, and hemostatic activation [3, 19, 20]. Impaired blood flow and ischemia occurs due to increased plasma viscosity from chronic hemolysis and sickle cell deformity. Due to the sickle cell form, the erythrocytes can be sequestered and promote transient vaso-occlusion. With the endothelial dysfunction and damage, there is upregulation of selectins, vascular-cell-adhesion-molecule −1, interleukin −8 on endothelial cells [3, 19, 20]. The increase in inflammatory markers, can increase neutrophil, monocyte and platelet activation and adhesion on damaged endothelium. Sequestering platelets causing thrombocytopenia is a predictor of progression of vaso-occlusion crisis to potentially lethal lung injury known as acute chest syndrome [3].

Acute chest syndrome is a complication of vaso-occlusive crisis and occurs 2–3 days after onset of vaso-occlusive pain [3, 19, 20]. When left untreated, it has a high mortality rate. It is caused by a hyper-inflammatory event, which usually in children is stimulated by an infection, that leads to release of cytokines, recruitment of neutrophils, breakdown of the endothelial-epithelial barrier, disruption of the oxygen exchange and acute lung injury.

Along with the vaso-occlusion, chronic hemolysis especially at higher rates cause progressive end-organ complications. Chronic anemia causes increase in cardiac output, dilation in ventricular chamber, and increase ventricular wall stress [3, 19]. Intravascular hemolysis causes endothelial damage from the increase reaction of oxy-Hb with nitric oxide forming inert nitrate which impairs nitric oxide dependent vasodilation [3, 19]. Also, with increase hemolysis, cell-free Hb promotes reactive oxygen species formation [3, 19].

Three treatment modalities are currently available: hydroxyurea, erythrocyte transfusion, and hematopoietic stem cell transplantation [3, 19, 20]. Hydroxyurea is a ribonucleotide reductase inhibitor that increases HbF expression and decreases leukocyte count [21]. Hydroxyurea decreases number of vaso-occlusive crises, hospitalizations and mortality in high-income countries. Another treatment is RBC transfusion [20]. This therapy improves microvascular flow due to decrease in circulating sickled RBCs. Continued transfusions can prevent stroke and vaso-occlusive crises. Complications though from regular transfusions include iron overload, alloimmunization, and hemolytic transfusion reactions. Current curative option for beta thalassemia major and sickle cell disease is allogenic hematopoietic stem cell transplantation if a human leukocyte antigen compatible donor is available [1]. If one is not available, ex-vivo gene therapy using autologous hematopoietic stem cells is considered a possible curative option [1, 22]. Ex-vivo lentiviral transfer to hematopoietic stem cells was a breakthrough in gene therapy curing a sickle cell patient [23].

Summary

Hemoglobin is a tetrameric protein composed of four globin chains. Each globin chain has a heme molecule with a central iron ion. The predominant hemoglobin in adults is Hemoglobin A which consists of two alpha and two beta globin subunits. Minor hemoglobin is present including Hemoglobin F which are two alpha and two gamma globin chains and Hemoglobin A2 which consists of two alpha and two delta subunits. Each heme group can bind to gases for transport. When a heme group binds to oxygen, it causes a structural change in hemoglobin from a tense state to a relaxed state. The relaxed state has an increased affinity for oxygen to the other heme molecules. The oxyhemoglobin dissociation curve can help visualize how different factors can affect the affinity of hemoglobin for oxygen.

Hemoglobinopathies are the most common monogenic disease. They occur when there are mutations affecting the hemoglobin chain structure or production and expression. Individuals with hemoglobinopathies can have varied expression of hemoglobin F and A2 due to the decrease or mutated expression of Hemoglobin A. Thalassemias occur when there are mutations in the hemoglobin chain affecting expression and production. Clinical presentation can vary from mild anemia to hydrops fetalis depending on the production and expression of the affected hemoglobin chain. Sickle cell disease is the most common form of hemoglobinopathies affecting hemoglobin chain structure. Though protective against malaria, individuals with sickle cell disease can have varied symptoms mostly due to vaso-occlusive propensity of the sickled erythrocytes.

Key Points

- Hemoglobin is produced in erythroid cells in the bone marrow of long bones and flat bones
- Hemoglobin is a tetrameric protein composed of four globin chains and each chain contains a heme molecule with a central iron ion
- Hemoglobin exists in two states: a tense deoxygenated state and a relaxed oxygenated state
- Hemoglobin is an allosteric protein therefore when one heme molecule binds to oxygen, hemoglobin transitions from its tense state to its relaxed state which increases its affinity for oxygen.
- Heme also binds to other gasses which allows for transport of these gasses to different tissues
- The oxyhemoglobin dissociation curve helps describe the relationship between oxygen saturation and partial pressure of oxygen.
- Thalassemias are a group of autosomal recessive disorders caused by decreased expression of either alpha or beta globin chains due to mutation or deletion in the alpha or beta gene
- Individuals with thalassemia can have a range of severity of symptoms based on the if the gene on both chromosomes is affected.
- Hemoglobinopathy occurs when there is a mutation causing a change in hemoglobin chain structure.
- Sickle Cell disease is due to an amino acid substitution in the beta globin chain causing hemoglobin to polymerize and the red blood cell to sickle
- Individuals with sickle cell disease have a propensity for blood vessel occlusion and ischemia leading to vaso-occlusive crisis.
- Current treatments available for sickle cell disease include hydroxyurea, red blood cell transfusion, and hematopoietic stem cell transplantation.

References

1. Farashi S, Harteveld CL. Molecular basis of alpha-thalassemia. Blood Cells Mol Dis. 2018;70:43–53.
2. Thomas C, Lumb AB. Physiology of haemoglobin. Continuing Educ Anaesth Crit Care Pain. 2012;12(5):251–6.
3. Kato GJ, et al. Sickle cell disease. Nat Rev Dis Primers. 2018;4:18010.
4. Marshall WJ, et al. Clinical biochemistry : metabolic and clinical aspects. 3rd ed. Edinburgh; New York: Churchill Livingstone/Elsevier; 2014. x, 932 pages.
5. Helms C, Kim-Shapiro DB. Hemoglobin-mediated nitric oxide signaling. Free Radic Biol Med. 2013;61:464–72.
6. Quaye IK. Extracellular hemoglobin: the case of a friend turned foe. Front Physiol. 2015;6:96.
7. Patel S, Jose A, Mohiuddin SS. Physiology, oxygen transport and carbon dioxide dissociation curve. Treasure Island: StatPearls; 2020.
8. Gell DA. Structure and function of haemoglobins. Blood Cells Mol Dis. 2018;70:13–42.
9. Benner A, et al. Physiology, Bohr effect. Treasure Island: StatPearls; 2020.
10. Origa R. beta-Thalassemia. Genet Med. 2017;19(6):609–19.
11. Kuesap J, et al. Coexistence of malaria and thalassemia in malaria endemic areas of Thailand. Korean J Parasitol. 2015;53(3):265–70.
12. Chapin JAG, Patricia J. Thalassemia syndromes. In: Hoffman R, editor. Hematology: basic principles and practice. Philadelphia: Elsevier; 2018. p. 546–70.
13. Farashi S, Najmabadi H. Diagnostic pitfalls of less well recognized HbH disease. Blood Cells Mol Dis. 2015;55(4):387–95.
14. Muncie HL Jr, Campbell J. Alpha and beta thalassemia. Am Fam Physician. 2009;80(4):339–44.
15. Vichinsky EP. Clinical manifestations of alpha-thalassemia. Cold Spring Harb Perspect Med. 2013;3(5):a011742.
16. Sabath DE. Molecular diagnosis of thalassemias and hemoglobinopathies: an ACLPS critical review. Am J Clin Pathol. 2017;148(1):6–15.
17. Viprakasit V, Ekwattanakit S. Clinical classification, screening and diagnosis for thalassemia. Hematol Oncol Clin North Am. 2018;32(2):193–211.
18. Fucharoen S, Weatherall DJ. The hemoglobin E thalassemias. Cold Spring Harb Perspect Med. 2012;2(8):1–15.
19. Sundd P, Gladwin MT, Novelli EM. Pathophysiology of sickle cell disease. Annu Rev Pathol. 2019;14:263–92.
20. Piel FB, Steinberg MH, Rees DC. Sickle cell disease. N Engl J Med. 2017;377(3):305.

21. Mangin O. High oxygen affinity hemoglobins. Rev Med Interne. 2017;38(2):106–12.
22. Coquerelle S, et al. Innovative curative treatment of beta thalassemia: cost-efficacy analysis of gene therapy versus allogenic hematopoietic stem-cell transplantation. Hum Gene Ther. 2019;30(6):753–61.
23. Ribeil JA, et al. Gene therapy in a patient with sickle cell disease. N Engl J Med. 2017;376(9):848–55.

The Global Burden of Anemia

5

Matthew A. Warner and Angela C. Weyand

Introduction

Anemia, literally translated from the Greek roots of "an" (without) and "haima" (blood), represents a decrease in the number of functional circulating erythrocytes, and therefore is most accurately assessed through measurement of red blood cell (RBC) mass. Unfortunately, such measurements are cumbersome and are unavailable in most clinical settings. As such, medical professionals are forced to rely on surrogates, including hemoglobin concentrations (i.e. the average amount of hemoglobin in a given volume of blood) and hematocrit (i.e. the volume of whole blood which is comprised by red cells, expressed as a percentage). While these are imperfect instruments influenced by changes in intravascular volume status and prone to laboratory errors, they are ubiquitously available in modern medical practice and generally provide a reasonable representation of true RBC or hemoglobin mass in the absence of acute bleeding [1]. Hence, anemia is typically defined by a hemoglobin concentration or hematocrit value falling below a reference normal value. In practical terms, anemia can be described as insufficient hemoglobin content (i.e. oxygen carrying capacity) to meet tissue oxygen demands.

With regards to hemoglobin concentrations, lower threshold values to define normality have been published in multiple investigations (Table 5.1) with the World Health Organization (WHO) defining anemia by a hemoglobin <13 g/dL in adult men and <12 g/dL in non-pregnant adult women [2]. While likely a reflection of multiple underlying physiological processes, sex differences in adult hemoglobin concentrations are thought to be driven, in part, by stimulatory effects of androgens on the bone marrow [3, 4]. Notably, normative hemoglobin values are derived from studies of distinct populations, which are limited in size and not representative of the entire global population. Indeed, numerous population-based investigations have noted disparate hemoglobin thresholds at which to define anemia [5]. Further, traditional anemia definitions are based on the observed distributions of hemoglobin concentrations in a given population of interest and not on the physiological significance of the hemoglobin concentrations themselves, though previous work has advocated for the importance of linking hemoglobin concentration-based anemia definitions to clinical outcomes [6–8].

Table 5.1 Hemoglobin concentrations utilized to define anemia across various studies

Source	Men g/dL	Women g/dL	Percent normal below cutoff
WHO (Blanc[1])	13	12	Not provided
Jandl[3]	14.2	12.2	2.5
Williams(Beutler[4])	14	12.3	2.5
Wintrobe(Lee[5])	13.2	11.6	Not provided
Rapaport[6]	14	12	Not provided
Goyette[7]	13.2	11.7	5
Tietz[8]	13.2	11.7	Not provided
Hoffman[9]	13.5	12	2.5

Adapted from Beutler [63, 64]
Lower limits of normal of hemoglobin concentration of the blood of adult men and women as assessed by various sources

It has been described that hemoglobin concentrations are lower in certain ethnic groups and races (i.e. Black compared to White persons) [9–11], which has generally been attributed to a higher prevalence of hemoglobinopathies (e.g. thalassemia trait) and other non-genetic factors such as chronic disease states and iron deficiency in Black persons. Yet, even after attempts to account differences in hemoglobinopathies, iron deficiency, and disease burden, persistent racial differences in hemoglobin concentrations remain [12–14], which have culminated in the creation of different reference standards to define anemia across races [15]. Importantly, low

M. A. Warner (✉)
Division of Critical Care, Department of Anesthesiology and Perioperative Medicine, Mayo Clinic, Rochester, MN, USA
e-mail: Warner.Matthew@mayo.edu

A. C. Weyand
Division of Pediatric Hematology and Oncology, Department of Pediatrics, University of Michigan, Ann Arbor, MI, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_5

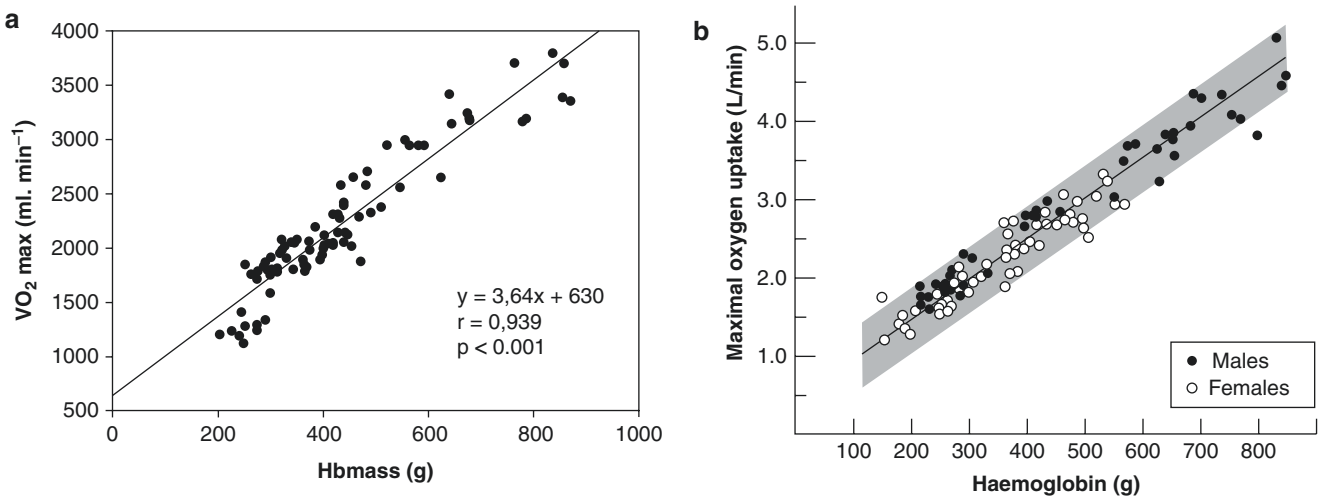


Fig. 5.1 Relationship between hemoglobin mass and maximal oxygen uptake (VO2 max) in children (**a**) and adults (**b**). (Adapted **a** from Prommer et al. [65] and **b** from Otto et al. [66])

hemoglobin concentrations are associated with increased mortality in both Black and White persons [6, 16], and attempts to utilize lower normative hemoglobin concentrations in Black persons are likely ill-conceived. Beyond the previously mentioned factors that may influence hemoglobin distributions across races, there are likely significant social determinants of health (i.e. inferior health care access and quality, differences in dietary habits, living conditions, and nutritional status, lower wages, structural racism), which have facilitated and perpetuated the acceptance of lower normative hemoglobin concentrations in Black persons. While these important determinants of health are often difficult to accurately assess in population-based studies, future research is clearly warranted to determine the exact drivers of differences in hemoglobin concentrations across races. For now, it is most sensible that we commit to employing consistent hemoglobin thresholds to define anemia irrespective of race.

Beyond race, hemoglobin values tend to decrease with age, though the precise physiological or pathologic drivers remain unclear [17]. Decreases in hemoglobin with age are generally greater in men than women such that sex-discrepancies in hemoglobin concentrations decrease with advancing age. Additionally, hemoglobin values are impacted by altitude [18, 19], with those living and/or training at higher elevations having increased hemoglobin mass in response to hypoxemia-driven increases in erythropoiesis. Other hypoxemia-inducing environmental factors (e.g. cigarette smoking) are also known to increase RBC production. Conversely, endurance athletes have been reported to have lower hemoglobin concentrations than the population average, which is likely multifactorial, including dilutional effects from increased plasma volumes [20], occult gastrointestinal bleeding with iron deficiency [21], intravascular hemolysis from repetitive trauma [22–24], and exercise-induced stimulation of pro-inflammatory cytokines, including hepcidin upregulation, with subsequent impairment of iron transport and erythropoiesis [25]. Conversely, the use of performance enhancing substances, including erythropoietin and steroids, may result in polycythemia in athletes. It is important to note that hemoglobin mass is strongly and positively correlated with maximal aerobic performance in both children [26] and adults [27–29], with each 1 gram increase in hemoglobin mass associated with a 4 ml/min increase in maximum oxygen consumption (i.e. VO_2max; Fig. 5.1).

Anemia Development and Classification

In broad terms, anemia can be secondary to one of three processes: blood loss, impaired RBC production, or premature destruction of RBCs. These mechanisms may occur alone or concurrently in any given patient. Blood loss may result in either rapid or slow development of anemia over time depending on the magnitude, speed, and frequency of bleeding. Impaired RBC production is typically related to nutritional deficiencies including iron, vitamin B12, and folate deficiencies, disease or drug-related bone marrow failure or suppression, inflammatory states characterized by impaired iron availability and/or reductions in the trophic hormones (e.g. erythropoietin, thyroxin, testosterone) that are responsible for the stimulation of RBC production (i.e. erythropoiesis), and disease-related reductions in trophic hormones such as that seen in end-stage renal disease, severe hypothyroidism, and gonadal disorders. Destruction of RBCs at a rate exceeding a typical 100 to 120-day RBC lifespan is used to define hemolysis. Hemolysis may occur intravascularly (i.e. in the vascular space itself) or more commonly in extravascular locations (e.g. liver, spleen). It may be secondary to

either congenital or acquired conditions. Further, hemolysis may result from mechanical devices such as prosthetic heart valves and ventricular assist devices. Hemolysis is often suspected based upon clinical findings (e.g. dark urine, jaundice) and laboratory evaluations (e.g. increased lactate dehydrogenase levels, reduced haptoglobin levels, increased indirect bilirubin levels, hemoglobinuria, hemoglobinemia).

Anemia may also be defined by the speed of development. In a general sense, anemia either develops rapidly (i.e. acute) or slowly (i.e. chronic) over time. The most common cause of acute anemia is blood loss, including traumatic and non-traumatic hemorrhage, surgical and procedural bleeding, and iatrogenic blood loss from phlebotomy [30–32]. Importantly, hemoglobin concentrations do not provide an accurate assessment of red cell mass or tissue oxygenation during acute bleeding [33–35]. Other notable causes of acute anemia include acute hemolysis, acute inflammation, medication, or infection-induced bone marrow suppression, and hemodilution, though the latter represents a state of plasma volume excess rather than actual hemoglobin deficiency [1]. Chronic anemia is characterized by slow loss of RBC mass over time and is most often associated with chronic medical conditions (i.e. chronic kidney disease, cancer, heart failure, chronic inflammatory or infectious states) or nutritional deficiencies. Iron deficiency remains the leading global nutritional deficiency resulting in anemia and the most common cause of anemia overall [36]. It is especially prevalent in persons residing in low- and middle-income countries, children, and women of child-bearing age [37, 38]. Iron deficiency, which often occurs without anemia, is primarily driven by insufficient dietary intake, impaired intestinal absorption, or recurrent episodes of blood loss. Folate and vitamin B12 deficiencies are other nutritional causes that may result in anemia development, typically developing over a period of months to years. Anemia, irrespective of chronicity, has consistently been associated with poor health outcomes.

Anemia may be further classified based upon morphological evaluation, most commonly RBC size. A normal RBC has a volume of approximately 80–100 femtoliters (fL). This reference range can therefore be utilized to facilitate evaluations for anemia etiology based upon mean corpuscular volumes (MCV) of RBCs falling below, within, or above the expected range. When the MCV exceeds 100 fL, anemia is macrocytic. Common causes of macrocytic anemia include folate and vitamin B12 deficiencies, alcohol abuse, chronic liver disease, hypothyroidism, and diseases associated with abnormal RBC maturation, including acute leukemia and myelodysplastic syndrome. Reticulocytes or immature RBCs are larger than mature RBCs so any condition with reticulocytosis can cause macrocytosis. Microcytic anemias are defined by an MCV less than 80 fL. Common causes include iron deficiency anemia, disorders of globin production (i.e. alpha and beta thalassemia), and disorders of heme synthesis (i.e. sideroblastic anemias). Normocytic anemias by definition have a normal MCV. It is important to consider that the MCV is generally reported through the use of an automatic cell counter on a peripheral blood smear. Hence, the MCV represents the average or typical RBC volume, and there may indeed exist subpopulations of RBCs that are microcytic or macrocytic; hence, a normal MCV does not exclude the possibility for an underlying microcytic or macrocytic anemia. In general, normocytic anemias are reflective of underlying systemic disorders associated with impairments in erythropoiesis.

Anemias occurring in hospitalized patients warrant special attention. As anemia is common in community-dwelling persons, it is perhaps not surprising that anemia is also common in those requiring hospitalization. However, many patients that do not have a previous diagnosis of anemia develop incident anemia during hospitalization (i.e. hospital-acquired anemia [HAA]). HAA is thought to occur in 33–75% of all hospitalized patients without a known history of anemia [39, 40]. The causes for HAA are multifactorial, but are most commonly related to blood loss, hemodilution, and impaired RBC production. Blood loss occurs as a result of hemorrhage, surgical and procedural bleeding, and iatrogenic blood loss through phlebotomy. Every 50 ml of phlebotomized blood is associated with a nearly 20% increase in the risk for developing moderate-to-severe HAA [31]. Hemodilution is defined by expansion of the circulating plasma volume without concurrent growth in hemoglobin mass, thereby culminating in a reduction in the hemoglobin concentration. Although not a true anemia per se, hemodilution creates an apparent anemia for clinicians who are accustomed to making anemia-treatment decisions (e.g. RBC transfusion) based upon threshold hemoglobin concentrations. It is important to recognize that the treatment for hemodilution-related anemia should be a reduction in the expanded plasma volume (e.g. diuresis) rather than further blood volume expansion with RBC transfusion. Finally, hospitalized patients are at heightened risk for impairments in RBC production. For example, inflammatory and infectious conditions result in the production of inflammatory cytokines which may result in decreased iron availability and decreases in erythropoietin [41, 42]. Critically ill patients and those requiring prolonged hospitalization are at particular risk for HAA [43, 44]. This anemia may persist long after hospitalization [41, 43] and has been associated with impairments of post-hospitalization physical function [45].

Health and Societal Implications of Anemia

Anemia has tremendous global impact. Approximately 2 billion people, more than ¼ of the global population, are estimated to be living with anemia [38]. The highest rates of anemia are found in low- and middle-income countries, with vast discrepancies across geographic regions (Fig. 5.2). For

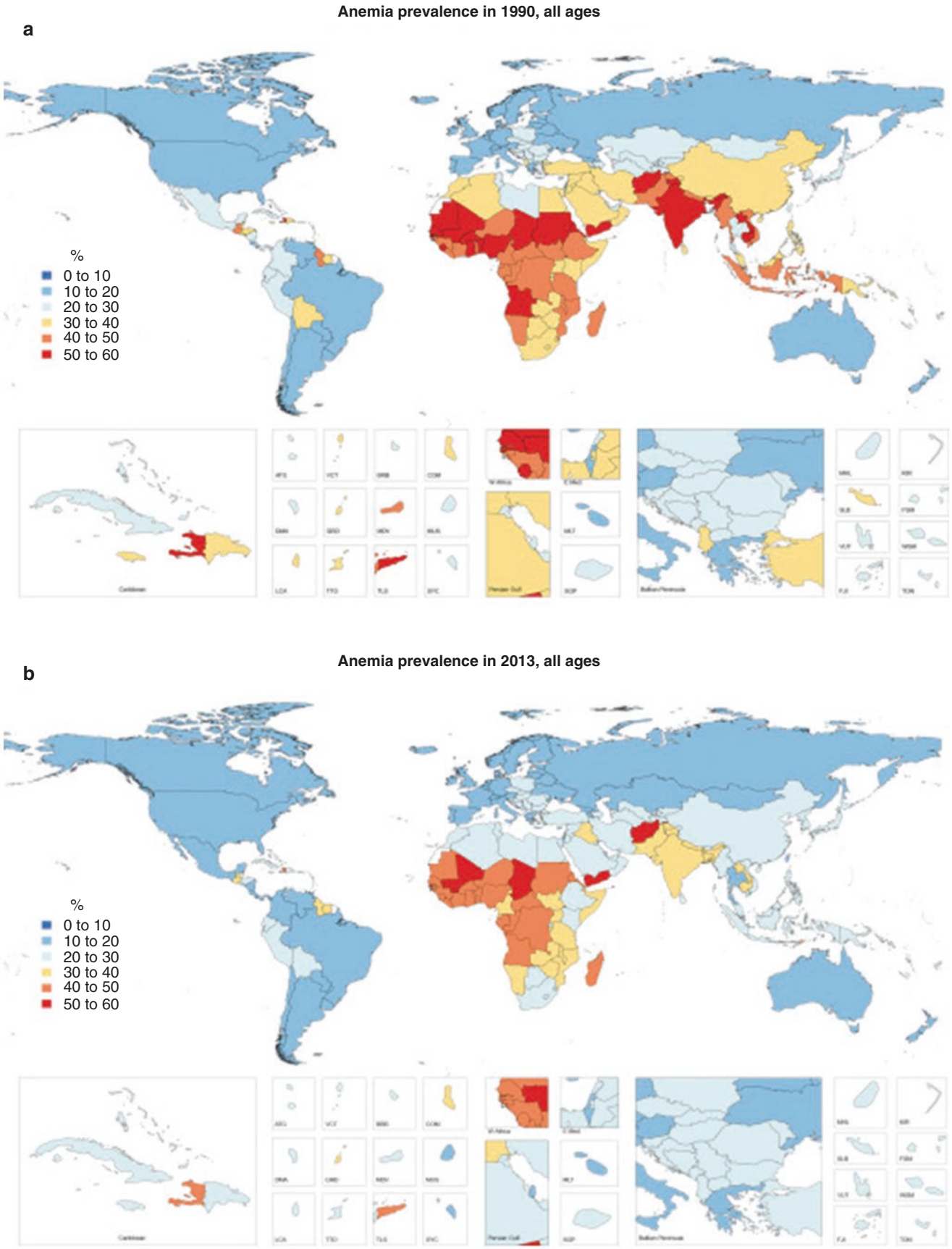


Fig. 5.2 Differences in anemia prevalence across the globe in 1990 (**a**) and 2013 (**b**). (Adopted from Kassebaum [67])

example, the prevalence of anemia in Sub-Saharan African is approximately 50,000–60,000 per 100,000 persons, which is substantially higher than a prevalence of 15,000 per 100,000 persons in Western Europe and the United States [37]. Iron deficiency, the leading cause of anemia globally, affects approximately 1 in every 5 women and 1 in every 7 men [37]. However, causes of anemia vary by geographic location, age, and sex [37, 38]. Notably, iron deficiency anemia and anemia of inflammation often coexist in low- and middle-income countries secondary to a high prevalence of nutritional deficiencies and chronic infections. Fortunately, anemia seems to be improving over time, with an estimated global prevalence of 27% in 2013 down from 33% in 1990 [38], though rates remain alarmingly high.

Anemia has consistently been associated with poor health outcomes, including decreased health-related quality of life and survival [46]. As red blood cells are the dominant vehicles for oxygen delivery to vital organs and peripheral tissue beds, it is perhaps not surprising that anemia is associated with a host of symptoms linked to inadequate tissue oxygen delivery. These include fatigue, weakness, impaired cognition, and concentration difficulties, though symptomatology may vary based on chronicity and degree of physiological compensation. Anemia symptoms may result in difficulties in school, decreased work productivity, diminished physical functioning, and impaired quality of life. Globally, it has been estimated that anemia is responsible for more than 68 million years lived with disability, which accounts for nearly 10% of total global disability from all causes [37]. This burden falls disproportionately on women and children. Further, anemia in women of childbearing age is associated with increased risk for preterm labor, low birth weight infants, and both maternal and infant mortality [47, 48]. This further aggravates global socioeconomic and health disparities.

In hospitalized patients, anemia has been associated with organ dysfunction such as acute kidney injury and myocardial ischemia [49, 50], prolonged hospital stays [39], mortality [39, 40, 51], readmissions [40, 52], and increasing hospital costs [39]. In surgical patients, anemia is similarly associated with poor perioperative outcomes, including longer hospital lengths of stay, increased transfusion requirements, postoperative organ dysfunction, and mortality [50, 53–59]. Despite this, only a fraction of patients receive dedicated evaluation and management of anemia prior to elective surgery; more than a quarter of patients proceed to surgery with untreated anemia [43]. Further, up to 90% of patients experience anemia following surgery [53]. Importantly, anemia is the leading predictor for red blood cell (RBC) transfusion in hospitalized patients, with transfusion itself being associated with a myriad of adverse clinical outcomes. While clinical trials have consistently demonstrated that more restrictive RBC transfusion practices (i.e. transfusion for hemoglobin <7–8 g/dL) are generally equivalent or superior to more liberal practices (i.e. transfusion for hemoglobin <9–10 g/dL) [60, 61], many clinicians continue to view allogenic transfusion as a primary treatment for anemia in the hospital setting [62]. Unfortunately, anemia is often ignored, or "tolerated", until hemoglobin concentrations fall below a given transfusion threshold, rather than evaluating and addressing the underlying causes.

Summary

Anemia is a common disorder defined by a decrease in the number of circulating erythrocytes, which generally occurs as a result of one (or more) of the following processes: blood loss, impaired red cell production, or premature red cell destruction. The effects of anemia are felt globally, yet this burden falls disproportionately upon those in lower and middle-income countries. Despite being associated with a host of adverse clinical outcomes, anemia is often ignored until it is deemed severe enough to warrant transfusion.

Key Points

- Anemia represents insufficient hemoglobin content to meet tissue oxygen demands and is typically defined by a hemoglobin concentration less than 13 g/dL in adult men and less than 12 g/dL in adult, non-pregnant women.
- Anemia may be defined by the underlying pathological processes that lead to its development, the speed of development, and by laboratory and morphological features.
- Iron deficiency is the leading cause of global anemia, which is particularly common in children and women.
- Anemia is experienced by more than 25% of the global population and is estimated to account for approximately 10% of global disability, with the greatest burden experienced in low- and middle-income countries.
- Symptoms of anemia include fatigue, concentration difficulties, and impaired aerobic capacity with subsequent impairments in quality of life.
- Anemia is associated with increased morbidity and mortality and is the leading risk factor for allogeneic red blood cell transfusion.

References

1. Otto JM, Plumb JOM, Clissold E, et al. Hemoglobin concentration, total hemoglobin mass and plasma volume in patients: implications for anemia. Haematologica. 2017;102(9):1477–85. https://doi.org/10.3324/haematol.2017.169680.
2. WHO Scientific Group on Nutritional Anaemias & World Health Organization. (1968). Nutritional Anaemias: Report of a WHO Scientific Group [meeting Held in Geneva from 13 to 17 March 1967]. World Health Organization.

3. Murphy WG. The sex difference in haemoglobin levels in adults-Mechanisms, causes, and consequences. Published online 2014. https://doi.org/10.1016/j.blre.2013.12.003.
4. Kennedy BJ, Gilbertsen AS. Increased erythropoiesis induced by androgenic-hormone therapy. N Engl J Med. 1957;256(16):719–26. https://doi.org/10.1056/nejm195704182561601.
5. Beutler E, Waalen J. The definition of anemia: what is the lower limit of normal of the blood hemoglobin concentration? Blood. 2006;107(5):1747–50. https://doi.org/10.1182/blood-2005-07-3046.
6. Zakai NA, Katz R, Hirsch C, et al. A prospective study of anemia status, hemoglobin concentration, and mortality in an elderly cohort: the cardiovascular health study. Arch Intern Med. 2005;165(19):2214–20. https://doi.org/10.1001/archinte.165.19.2214.
7. Culleton BF, Manns BJ, Zhang J, Tonelli M, Klarenbach S, Hemmelgarn BR. Impact of anemia on hospitalization and mortality in older adults. Blood. 2006;107(10):3841–6. https://doi.org/10.1182/blood-2005-10-4308.
8. Ripoll JG, Smith MM, Hanson AC, et al. Sex-specific associations between preoperative anemia and postoperative clinical outcomes in patients undergoing cardiac surgery. Anesth Analg. 2021; https://doi.org/10.1213/ANE.0000000000005392.
9. Dong X, de Leon CM, Artz A, Tang Y, Shah R, Evans D. A population-based study of hemoglobin, race, and mortality in elderly persons. J Gerontol Ser A Biol Sci Med Sci. 2008;63(8):873–8. https://doi.org/10.1093/gerona/63.8.873.
10. Johnson-Spear MA, Yip R. Hemoglobin difference between black and white women with comparable iron status: justification for race-specific anemia criteria. Am J Clin Nutr. 1994;60(1):117–21. https://doi.org/10.1093/ajcn/60.1.117.
11. Jackson RT. Separate hemoglobin standards for blacks and whites: a critical review of the case for separate and unequal hemoglobin standards. Med Hypotheses. 1990;32(3):181–9. https://doi.org/10.1016/0306-9877(90)90121-T.
12. Beutler E, West C. Hematologic differences between African-Americans and whites: the roles of iron deficiency and α-thalassemia on hemoglobin levels and mean corpuscular volume. Blood. 2005;106(2):740–5. https://doi.org/10.1182/blood-2005-02-0713.
13. Perry GS, Byers T, Yip R, Margen S. Iron nutrition does not account for the hemoglobin differences between blacks and whites. J Nutr. 1992;122(7):1417–24. https://doi.org/10.1093/jn/122.7.1417.
14. Zakai NA, McClure LA, Prineas R, et al. Correlates of anemia in American blacks and whites: the REGARDS Renal Ancillary study. Am J Epidemiol. 2009;169(3):355–64. https://doi.org/10.1093/aje/kwn355.
15. Lim E, Miyamura J, Chen JJ. Racial/ethnic-specific reference intervals for common laboratory tests: a comparison among Asians, Blacks, Hispanics, and White. Hawaii J Med Public Health. 2015;74(9):302–10. www.cdc.gov/nchs/nhanes.htm. Accessed 21 Jan 2021.
16. Denny SD, Kuchibhatla MN, Cohen HJ. Impact of anemia on mortality, cognition, and function in community-dwelling elderly. Am J Med. 2006;119(4):327–34. https://doi.org/10.1016/j.amjmed.2005.08.027.
17. Stauder R, Valent P, Theurl I. Anemia at older age: etiologies, clinical implications, and management. Blood. 2018;131(5):505–14. https://doi.org/10.1182/blood-2017-07-746446.
18. Strømme SB, Ingjer F. High altitude training. Nord Med. 1994;109(1):19–22. https://doi.org/10.2165/00007256-199214050-00002.
19. Heinicke K, Heinicke I, Schmidt W, Wolfarth B. A three-week traditional altitude training increases hemoglobin mass and red cell volume in elite biathlon athletes. Int J Sports Med. 2005;26(5):350–5. https://doi.org/10.1055/s-2004-821052.
20. Sawka MN, Convertino VA, Eichner ER, Schnieder SM, Young AJ. Blood volume: importance and adaptations to exercise training, environmental stresses, and trauma/sickness. Med Sci Sports Exerc. 2000;32(2):332–48. https://doi.org/10.1097/00005768-200002000-00012.
21. Rudzki SJ, Hazard H, Collinson D. Gastrointestinal blood loss in triathletes: It's etiology and relationship to sports anaemia. Aust J Sci Med Sport. 1995;27(1):3–8. Accessed 18 Dec 2020. https://europepmc.org/article/med/7780774.
22. Dufaux B, Hoederath A, Streitberger I, Hollmann W, Assmann G. Serum ferritin, transferrin, haptoglobin, and iron in middle- and long-distance runners, elite rowers, and professional racing cyclists. Int J Sports Med. 1981;2(1):43–6. https://doi.org/10.1055/s-2008-1034583.
23. Haymes EM, Lamanca JJ. Iron loss in runners during exercise implications and recommendations. Sport Med. 1989;7(5):277–85. https://doi.org/10.2165/00007256-198907050-00001.
24. Lippi G, Sanchis-Gomar F. Epidemiological, biological and clinical update on exercise-induced hemolysis. Ann Transl Med. 2019;7(12):270. https://doi.org/10.21037/atm.2019.05.41.
25. Peeling P, Dawson B, Goodman C, Landers G, Trinder D. Athletic induced iron deficiency: new insights into the role of inflammation, cytokines and hormones. Eur J Appl Physiol. 2008;103(4):381–91. https://doi.org/10.1007/s00421-008-0726-6.
26. Prommer N, Wachsmuth N, Thieme I, et al. Influence of endurance training during childhood on total hemoglobin mass. Front Physiol. 2018;9(MAR) https://doi.org/10.3389/fphys.2018.00251.
27. Schmidt W, Prommer N. Impact of alterations in total hemoglobin mass on V˙O2max. Exerc Sport Sci Rev. 2010;38(2):68–75. https://doi.org/10.1097/JES.0b013e3181d4957a.
28. Otto JM, Montgomery HE, Richards T. Haemoglobin concentration and mass as determinants of exercise performance and of surgical outcome. Extrem Physiol Med. 2013;2(1):33. https://doi.org/10.1186/2046-7648-2-33.
29. Gore C, Hahn A, Burge C, Telford R. VO_2 max and haemoglobin mass of trained athletes during high intensity training. Int J Sports Med. 1997;28(06):477–82. https://doi.org/10.1055/s-2007-972667.
30. Thavendiranathan P, Bagai A, Ebidia A, Detsky AS, Choudhry NK. Do blood tests cause anemia in hospitalized patients? The effect of diagnostic phlebotomy on hemoglobin and hematocrit levels. J Gen Intern Med. 2005;20(6):520–4. https://doi.org/10.1111/j.1525-1497.2005.0094.x.
31. Salisbury AC, Reid KJ, Alexander KP, et al. Diagnostic blood loss from phlebotomy and hospital-acquired anemia during acute myocardial infarction. Arch Intern Med. 2011;171(18):1646–53. https://doi.org/10.1001/archinternmed.2011.361.
32. Chant C, Wilson G, Friedrich JO. Anemia, transfusion, and phlebotomy practices in critically ill patients with prolonged ICU length of stay: a cohort study. Crit Care. 2006;10(5):R140. https://doi.org/10.1186/cc5054.
33. Gutierrez G, Reines HD, Wulf-Gutierrez ME. Clinical review: hemorrhagic shock. Crit Care. 2004;8(5):373–81. https://doi.org/10.1186/cc2851.
34. Nelson DP, King CE, Dodd SL, Schumacker PT, Cain SM. Systemic and intestinal limits of O2 extraction in the dog. J Appl Physiol. 1987;63(1):387–94. https://doi.org/10.1152/jappl.1987.63.1.387.
35. Will ND, Kor DJ, Frank RD, et al. Initial postoperative hemoglobin values and clinical outcomes in transfused patients undergoing noncardiac surgery. Anesth Analg. 2019;129(3):819–29. https://doi.org/10.1213/ANE.0000000000004287.
36. Miller JL. Iron deficiency anemia: a common and curable disease. Cold Spring Harb Perspect Med. 2013;3(7) https://doi.org/10.1101/cshperspect.a011866.

37. Kassebaum NJ, Jasrasaria R, Naghavi M, et al. A systematic analysis of global anemia burden from 1990 to 2010. Blood. 2014;123(5):615–24. https://doi.org/10.1182/blood-2013-06-508325.
38. Kassebaum NJ. The global burden of anemia. https://doi.org/10.1016/j.hoc.2015.11.002.
39. Koch CG, Li L, Sun Z, et al. Hospital-acquired anemia: prevalence, outcomes, and healthcare implications. J Hosp Med. 2013;8(9):506–12. https://doi.org/10.1002/jhm.2061.
40. Makam AN, Nguyen OK, Clark C, Halm EA. Incidence, predictors, and outcomes of hospital-acquired anemia. https://doi.org/10.12788/jhm.2712.
41. Bateman AP, McArdle F, Walsh TS. Time course of anemia during six months follow up following intensive care discharge and factors associated with impaired recovery of erythropoiesis. Crit Care Med. 2009;37(6):1906–12. https://doi.org/10.1097/CCM.0b013e3181a000cf.
42. Hayden SJ, Albert TJ, Watkins TR, Swenson ER. Anemia in critical illness. Am J Respir Crit Care Med. 2012;185(10):1049–57. https://doi.org/10.1164/rccm.201110-1915ci.
43. Warner MA, Hanson AC, Frank RD, et al. Prevalence and recovery from anemia following hospitalization for critical illness among adults in Minnesota, 2010–2016. JAMA Netw Open. 2020;3(9):e2017843.
44. Corwin HL, Gettinger A, Pearl RG, et al. The CRIT study: anemia and blood transfusion in the critically ill – current clinical practice in the United States. Crit Care Med. 2004;32(1):39–52. https://doi.org/10.1097/01.CCM.0000104112.34142.79.
45. Warner MA, Kor DJ, Frank RD, et al. Anemia in critically ill patients with acute respiratory distress syndrome and posthospitalization physical outcomes. J Intensive Care Med. 1(9) https://doi.org/10.1177/0885066620913262.
46. Wouters HJCM, van der Klauw MM, de Witte T, et al. Association of anemia with health-related quality of life and survival: a large population-based cohort study. Haematologica. 2019;104(3):468–76. https://doi.org/10.3324/haematol.2018.195552.
47. Murphy JF, Newcombe RG, O'Riordan J, Coles EC, Pearson JF. Relation of haemoglobin levels in first and second trimesters to outcome of pregnancy. Lancet. 1986;327(8488):992–5. https://doi.org/10.1016/S0140-6736(86)91269-9.
48. Smith C, Teng F, Branch E, Chu S, Joseph KS. Maternal and perinatal morbidity and mortality associated with anemia in pregnancy. Obstet Gynecol. 2019;134(6):1234–44. https://doi.org/10.1097/AOG.0000000000003557.
49. Fowler AJ, Ahmad T, Abbott TEF, et al. Association of preoperative anaemia with postoperative morbidity and mortality: an observational cohort study in low-, middle-, and high-income countries. Br J Anaesth. 2018;121(6):1227–35. https://doi.org/10.1016/j.bja.2018.08.026.
50. Ranucci M, Di Dedda U, Castelvecchio S, Menicanti L, Frigiola A, Pelissero G. Impact of preoperative anemia on outcome in adult cardiac surgery: a propensity-matched analysis. Ann Thorac Surg. 2012;94(4):1134–41. https://doi.org/10.1016/j.athoracsur.2012.04.042.
51. Warner MA, Hanson AC, Frank RD, et al. Prevalence of and recovery from anemia following hospitalization for critical illness among adults. JAMA Netw Open. 2020;3(9):e2017843. https://doi.org/10.1001/jamanetworkopen.2020.17843.
52. Koch CG, Li L, Sun Z, et al. Magnitude of anemia at discharge increases 30-day hospital readmissions. J Patient Saf. 2017;13(4):202–6. https://doi.org/10.1097/PTS.0000000000000138.
53. Shander A, Knight K, Thurer R, Adamson J, Spence R. Prevalence and outcomes of anemia in surgery: a systematic review of the literature. Am J Med. 2004;116:58–69. https://doi.org/10.1016/j.amjmed.2003.12.013.
54. Warner MA, Shore-Lesserson L, Shander A, Patel SY, Perelman SI, Guinn NR. Perioperative anemia. Anesth Analg. 2020:1. https://doi.org/10.1213/ANE.0000000000004727.
55. Matzek LJ, Hanson AC, Schulte PJ, Evans KD, Kor DJ, Warner MA. The prevalence and clinical significance of preoperative thrombocytopenia in adults undergoing elective surgery: an observational cohort study. Anesth Analg. 2021; https://doi.org/10.1213/ane.0000000000005347.
56. Mueller MM, Van Remoortel H, Meybohm P, et al. Patient Blood Management: recommendations from the 2018 Frankfurt consensus conference. JAMA J Am Med Assoc. 2019;321(10):983–97. https://doi.org/10.1001/jama.2019.0554.
57. Foss NB, Kristensen MT, Kehlet H. Anaemia impedes functional mobility after hip fracture surgery. Age Ageing. 2008;37(2):173–8. https://doi.org/10.1093/ageing/afm161.
58. Faraoni D, Dinardo JA, Goobie SM. Relationship between preoperative anemia and in-hospital mortality in children undergoing noncardiac surgery. Anesth Analg. 2016;123(6):1582–7. https://doi.org/10.1213/ANE.0000000000001499.
59. Fowler AJ, Ahmad T, Phull MK, Allard S, Gillies MA, Pearse RM. Meta-analysis of the association between preoperative anaemia and mortality after surgery. Br J Surg. 2015;102(11):1314–24. https://doi.org/10.1002/bjs.9861.
60. Hébert PC, Wells G, Blajchman MA, et al. A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. N Engl J Med. 1999;340(6):409–17. https://doi.org/10.1056/NEJM199902113400601.
61. Carson JL, Stanworth SJ, Alexander JH, et al. Clinical trials evaluating red blood cell transfusion thresholds: an updated systematic review and with additional focus on patients with cardiovascular disease. Am Heart J. 2018;200 https://doi.org/10.1016/j.ahj.2018.04.007.
62. Shander A, Goodnough LT. From tolerating anemia to treating anemia. Ann Intern Med. 2019;170(2):125–6. https://doi.org/10.7326/M18-3145.
63. Beutler E. The definition of anemia: what is the lower limit of normal of the blood hemoglobin concentration. Blood. 2006;107(5):1747–50.
64. Blanc B, Finch CA, Hallberg L, et al. Nutritional anaemias. Report of a WHO Scientific Group. WHO Tech Rep Ser. 1968;405: 1-40. Jandl JH. Blood. Boston, MA: Little, Brown and Company; 1996. Beutler E, Lichtman MA, Coller BS, Kipps TJ, Seligsohn U. Williams Hematology. New York, NY: McGraw-Hill; 2001. Lee GR, Foerster J, Lukens J, Paraskevas F, Greer JP, Rodgers GM. Wintrobe's Clinical Hematology. Baltimore, MD: Williams and Wilkins; 1998 Rapaport SI. Introduction to Hematology. Philadelphia, PA: JB Lippincott Company; 1987. Goyette RE. Hematology. A Comprehensive Guide to the Diagnosis and Treatment of Blood Disorders. Los Angeles, CA: Practice Management Information Corporation (PMIC); 1997. Tietz NW. Clinical Guide to Laboratory Tests. Philadelphia, PA: WB Saunders Co; 1995. Hoffman R, Benz EJ Jr, Shattil SJ, Furie B. Hematology: Basic Principles and Practice. New York, NY: Churchill-Livingstone; 2004.
65. Prommer N, et al. Influence of endurance training during childhood on total hemoglobin mass. Front Physiol. 2018;9:251.
66. Otto J, et al. Haemoglobin concentration and mass as determinants of exercise performance and or surgical outcome. Extreme Physiol Med. 2013;2(1):art33, 1–8.
67. Kassebaum NJ. The global burden of anemia. Hematol Oncol Clin North Am. 2016;30(2):247–308.

Blood Component Therapy: The History, Efficacy, and Adverse Effects in Clinical Practice

6

William Davis, Amanda Frantz, Meghan Brennan, and Corey S. Scher

Introduction

Blood transfusions are a crucial, life-saving treatment for a range of disorders and are among the most common medical interventions in use today. In the United States and other developed countries, whole blood is rarely used therapeutically, however. Instead, blood is separated into its components during or immediately following its collection from a donor. This allows for each blood component to be processed and stored under optimal conditions. Platelets and certain coagulation factors quickly lose their clinical efficacy when stored in whole blood. When processed and stored separately from red blood cells, in contrast, platelets and factor-rich plasma retain their viability. In addition, the separation of blood components allows for each product to be transfused independently based upon the specific pathology being treated. Disorders of hemostasis, for example, may be treated with platelets, plasma, or specific coagulation factors. Symptomatic anemia, meanwhile, is managed with the transfusion of red cell concentrates.

In the setting of massive hemorrhage, patients are typically deficient in all blood components. Even in cases such as these, requiring transfusion of whole-blood equivalents, component therapy has been shown to be non-inferior to the transfusion of whole blood. As such, the use of whole blood is limited to a small number of specific indications that include neonatal exchange transfusions and some cardiovascular surgeries. While component therapy is nearly universal in wealthier countries, whole blood continues to be used in parts of the world where blood-banking infrastructure is underdeveloped.

W. Davis · C. S. Scher (✉)
NYU-Grossman School of Medicine, Department of Anesthesiology, New York, NY, USA
e-mail: William.Davis@nyulangone.org

A. Frantz · M. Brennan
University of Florida College of Medicine, Department of Anesthesiology, Gainesville, Florida, USA
e-mail: AFrantz@anest.ufl.edu; mbrennan@anest.ufl.edu

Blood components may be derived from whole-blood donations or collected directly by means of apheresis. In either case, whole blood is initially separated into its major components by density gradient centrifugation. The subsequent processing, preservation, and storage of each blood component is unique to the product being manufactured.

This chapter describes the basic characteristics, processing, storage of the major blood components. These include red blood cell concentrates, plasma-derived blood products, and platelets. The clinical use and physiologic effects of these blood products are also addressed.

Red Cell Components

Blood product components are collected from donors in the form of whole blood through apheresis, a method that first removes whole blood and filters out specific components and returning the remaining components to the donor. After collection, whole blood is biologically processed by centrifugation to separate red blood cells from platelets and plasma. The separation of whole blood into its cellular components, allows each to be processed and stored under optimal conditions allowing targeted therapeutic deployment. Blood banking, the preservation and long-term storage of blood components, is essential to maintaining an adequate store of available blood products and allows for blood product screening, testing, and processing prior to transfusion.

Red Blood Cell Concentrates

RBCs, administered in the form of red cell concentrate (RCC), are the most frequently transfused blood product. Common indications for transfusion include: acute blood loss anemia, symptomatic anemia, and sickle cell crisis. RCC transfusion volume generally consists of 350 ml and a hematocrit of approximately 60%. On average, transfusion of a single unit in an adult patient increases hemoglobin by 1 g/dL.

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_6

Collection and Processing

Whole blood is collected into bags containing a buffered, sodium-citrate anticoagulant solution, typically citrate, phosphate, dextrose or CPDA-1 (Table 6.1). The resulting mixture is then separated by centrifugation into RBCs and platelet-rich plasma. The concentrated red blood cells are isolated and then re-suspended in a preservation solution (Table 6.1). In order to manufacture platelets or fresh frozen plasma, whole blood must be separated into its components and processed within 8 hours of collection. If platelets are to be separated from the sample, the whole blood is maintained at room temperature prior to separation [1].

Depending upon the collection system used, a 450- or 500-ml volume of whole blood is collected from each donor. Assuming a donor with a hematocrit of 42%, 500 ml of whole blood contains approximately 210 ml of RBCs. During centrifugation, roughly 90% of both the plasma and anticoagulant solution is removed [2], bringing the hematocrit of the red-cell concentrate to between 80 and 90%. The added volume of the preservation solution dilutes the mixture, lowering the hematocrit to approximately 60% [3]. The final concentration of the RCC balances a desire to minimize the unit volume against the need to ensure adequate diffusion of nutrients during storage, which also limits the viscosity of the product at transfusion. Notably, the actual volume of RBCs in a unit of blood and the final hemoglobin content of the RCC varies depending upon the characteristics of the donor. In addition, the volume of RBCs may be reduced by up to 15% in units that are filtered to remove white blood cells (WBCs) [1].

While the vast majority of RCC units are derived from whole-blood donations, RBCs may also be collected by apheresis. In the US, approximately 15% of RCC units are now produced in this manner [4]. Apheresis allows for the collection of two RCC units from a single donor. If both units are administered to the same patient, this offers the benefit of limiting blood-donor exposure. Additionally, apheresis allows for the production of RCC units with consistent RBC content [5]. Modern apheresis devices are designed to collect a specific volume of RBCs, and thus yield a relatively standardized product. RBCs collected by apheresis have been shown to be of similar quality to those collected by conventional, whole-blood donation [6].

RCCs can generally be stored at 4 °C for up to 42 days, though variation of storage time exists depending upon the methods of processing, preservation solutions, institutional policies, and national regulatory guidelines. In Germany and Switzerland, for example, RCCs may be stored for up to 49 days using certain additives [7]. The national blood services in the UK and the Netherlands, meanwhile, limit the storage of RCCs to 35 days [7].

Table 6.1 A & B: Compositions of common anticoagulant and additive solutions (all concentrations in mmol/L) [2]

	CPD	CPDA-1
Citric acid	14	14
Sodium citrate	116	117
Monosodium phosphate	15.8	16
Adenine	0	2
Dextrose	141	142

	SAG-M	AS-1	AS-3	AS-5
NaCl	150	154	70	154
Phosphate	0	0	23	0
Adenine	1.25	2	2	2
Glucose	45	111	55	45
Mannitol	30	41.2	0	29
Citric acid	0	0	2	0
Sodium citrate	0	0	30	0

Principles of Preservation

In order to improve oxygen delivery to tissues, RBCs must survive storage and remain in circulation following transfusion. Thus, methods of preservation and storage have been developed to minimize the extent of *ex vivo* hemolysis and maximize the rate of *in vivo* recovery following transfusion. It has long been recognized that the gradual depletion of intracellular ATP during storage impairs the post-transfusion recovery of RBCs [8, 9]. Several components of the standard anticoagulant and additive solutions – including glucose, adenine, and phosphate – aid in preservation of ATP levels during storage and facilitate its rapid regeneration after transfusion.

Lacking a nucleus, mitochondria, and other organelles needed for energy production, RBCs produce ATP through the breakdown of glucose to lactate or pyruvate. Whole blood contains sufficient glucose to permit refrigerated storage for approximately 5 days [3]. Additive solutions are formulated to both replace the glucose lost with plasma removal and supplement the glucose supply to allow for longer-term storage [1].

A depletion of intracellular phosphate is also thought to impair the regeneration of ATP following transfusion. Phosphate slowly leaks from RBCs during storage due to the breakdown of 2,3-DPG in the acidic storage environment [10]. The supraphysiologic concentration of phosphate present in anticoagulant solutions slows its diffusion out of the cells, reducing the depletion of intracellular phosphate stores and modestly improving post-transfusion recovery [2]. Finally, anticoagulant and additive solutions contain adenine, which has been shown to promote the maintenance of ATP levels during prolonged storage [11]. Mannitol is the final major constituent of most additive solutions. Its use limits the osmotic swelling of RBCs and further reduces the

rate of hemolysis during storage [2]. Mannitol has the additional benefit of absorbing free radicals in the storage environment [12].

The type of container used to store RBCs is known to have a significant impact on rates of *ex vivo* hemolysis and *in vivo* recovery. Blood products are generally stored in polyvinyl chloride (PVC) bags. These are permeable to small gas molecules, which allows for the release of carbon dioxide generated by RBCs during glycolysis. This effect limits the fall in pH that occurs during storage. In addition, the diethylhexylphthalate (DEHP) plasticizer used in PVC diffuses into the stored blood, where it stabilizes the RBC membrane and minimizes membrane loss. Together, these factors significantly reduce the rate of hemolysis and allow for blood to be stored twice as long in PVC bags as in other plastic or glass containers [3].

Finally, the filtering of WBCs from collected blood prior to storage reduces *ex vivo* hemolysis and improves the rate of *in vivo* recovery after transfusion [13]. More recently, this has also been shown to attenuate a variety of oxidative storage lesions [14]. While leukoreduction has been widely adopted in the US and Europe, studies have yet to demonstrate the clinical efficacy of the practice [15].

RBC Storage Lesions

RBCs are stored under conditions that diverge markedly from those found in the human body. Most notably, RBCs are maintained at low temperatures and stored in a closed system that allows for the buildup of many metabolic byproducts. The result is the accumulation of biochemical and structural changes during storage. While some of these changes are reversible upon transfusion, others have lasting effects on cell function. Clinically, these lesions compromise the therapeutic efficacy of RCCs and contribute to many of the adverse outcomes associated with RBC transfusion.

Metabolic Impairment

Although metabolic activity is slowed under hypothermic conditions, glycolysis continues within RBCs, and lactate gradually accumulates in the storage medium. As a result, the pH at the start of storage medium falls from 7.05 to approximately 6.4 after 6 weeks [9]. The acidic nature of the storage environment reduces the activity of the rate-limiting enzymes in key metabolic pathways, resulting in the rapid depletion of 2,3-DPG and the gradual exhaustion of ATP [16]. Oxidative damage to these enzymes during storage further compromises their function. Notably, the availability of glucose is not a limiting factor for ATP generation due to the high concentrations of glucose in all of the widely used additive solutions (Fig. 2).

These metabolic impairments are largely reversed following transfusion. Intracellular pH normalizes and stores of ATP and 2,3-DPG are quickly regenerated. During storage, however, these metabolic deficiencies induce a cascade of biochemical and structural changes that are not easily reversed.

Oxidative Injury

RBCs are subjected to a significant degree of oxidative stress *in vivo* due to high intracellular concentrations of both ferrous iron and oxygen. As such, they have evolved elaborate mechanisms for protecting against oxidative injury. In the presence of oxygen, hemoglobin is slowly oxidized to methemoglobin, generating the reactive oxygen species (ROS) superoxide (O_2^-) as a byproduct (Eq. 6.1). Normally, methemoglobin is quickly reduced to hemoglobin by an NADH-dependent reductase. Superoxide is converted to hydrogen peroxide (H_2O_2) (Eq. 6.2), which is then eliminated through reaction with reduced glutathione (GSH) (Eq. 6.3) or dismutation by catalase (Eq. 6.4).

$$\mathrm{HbFe(II)O_2} \rightarrow \mathrm{HbFe(III)} + \mathrm{O_2^-} \tag{6.1}$$

$$2\,\mathrm{O_2^-} + 2\,\mathrm{H^+} \rightarrow \mathrm{H_2O_2} + \mathrm{O_2}\,(\text{superoxide dismutase}) \tag{6.2}$$

$$\mathrm{H_2O_2} \rightarrow 2\,\mathrm{H_2O}\,/\,\mathrm{GSH} \rightarrow \mathrm{GSSG}\,(\text{glutathione peroxidase}) \tag{6.3}$$

$$2\,\mathrm{H_2O_2} \rightarrow 2\,\mathrm{H_2O} + \mathrm{O_2}\,(\text{catalase}) \tag{6.4}$$

$$\mathrm{GSSG} + \mathrm{NADPH} + \mathrm{H^+} \rightarrow 2\,\mathrm{GSH} + \mathrm{NADP^+}\,(\text{glutathione reductase}) \tag{6.5}$$

The storage of RBCs under hypothermic conditions impairs these protective mechanisms. Additionally, the concentration of oxygen in the extracellular environment rises due its increased solubility at low temperatures. Both factors contribute to an increase in the susceptibility of RBCs to oxidative injury during storage.

The elimination of ROS generally involves the consumption of reducing equivalents such as GSH. In RBCs, these reducing equivalents are regenerated using NADPH (Eq. 6.5) produced via the ATP-dependent hexose monophosphate shunt. The depletion of ATP during storage, described above, inhibits the production of these electron donors and allows for an accumulation of oxidized hemoglobin and ROS over time. In its reduced form, GSH plays an essential role in the elimination of H_2O_2 (Eq. 6.3). During storage, however, there is a significant decline in both GSH levels [17] and the activity of related enzymes, including glutathione peroxidase and glutathione reductase [18]. The associated rise in H_2O_2 concentration is believed to be a significant mediator of oxidative injury [19]. While the autoxidation of hemoglobin to

methemoglobin is potentially reversible, subsequent oxidation by H_2O_2 can result in its irreversible degradation [19]. These reactions are accompanied by the generation of additional oxidizing species [20], while the release of heme from denatured hemoglobin causes oxidative injury to the cell membrane. Finally, the degradation heme releases free iron, which can generate hydroxyl radicals via the Fenton reaction [19] and thus drive further oxidative injury. Exposure to the plasticizer DEHP is another source of oxidative stress for stored RBCs. While DEHP is known to reduce the rate of membrane vesiculation during storage, it is also associated with an increase in lipid peroxidation and depletion of vitamin E and GSH [21]. Finally, the exposure of RBCs to supraphysiologic concentrations of glucose contributes to the nonenzymatic glycation of hemoglobin [22].

In studies of patients with diabetes, the glycation of hemoglobin is associated with an increase in ROS formation and lipid peroxidation [23].

The exposure of RBCs to high levels of oxidative stress during storage has a number of important functional consequences. Methemoglobin is unable to bind oxygen at physiologic concentrations, and thus the accumulation of oxidized hemoglobin impairs the ability of these cells to effectively deliver oxygen to tissues [24]. More significant, is the oxidation of cytoskeletal proteins and peroxidation of lipid membranes, which induces changes in the structural properties of stored RBCs.

Membrane Asymmetry

Normal RBCs are characterized by an asymmetrical distribution of phospholipids across their cell membranes: choline phospholipids localize to the outer leaflet, while aminophospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) localize to the inner leaflet [25]. Bilayer asymmetry is maintained by an ATP-dependent aminophospholipid translocase, which transports PS and PE from the outer to the inner leaflet [26], and is opposed by the activity of phospholipid scramblases, which mediate the bidirectional movement of phospholipids across the membrane.

The declines in intracellular ATP, potassium, and pH that occur during storage all act to inhibit aminophospholipid translocase [27, 28]. Under hypothermic storage conditions, however, the activity of all enzymes, including the phospholipid scramblases, is reduced. As a result, the loss of phospholipid asymmetry is limited while RBCs remain in storage. After 5 weeks, the fraction of RBCs expressing PS rises from approximately 0.5–1.5% [27]. In the 24 hours after transfusion, however, the loss of membrane asymmetry is thought to accelerate significantly with a restoration of scramblase activity [28]. This process is amplified by the intracellular accumulation of calcium during storage. The depletion of ATP impairs the energy-dependent efflux of calcium from RBCs, allowing its concentration to rise. Calcium is a key mediator of eryptosis and serves to activate phospholipid scramblases [29].

Following transfusion, the exposure of PS on the outer surface of RBCs marks these cells for phagocytosis by splenic and hepatic macrophages [28, 30]. The loss of membrane asymmetry thus impairs RBC recovery and likely contributes to the high rate of extravascular hemolysis after transfusion.

The externalization of PS is also thought to contribute to the procoagulant activity of transfused RBCs. The negatively charged PS serves as an assembly sites for coagulation complexes [31], binding factors II, V, VII, VIII, IX and X [29]. It has also been shown to be an essential cofactor for the prothrombinase enzyme complex [29]. As a result, PS exposure on RBC increases the rate of thrombin formation.

Additionally, the translocation of phosphatidylserine to the surface of RBCs during storage enhances the ability of transfused RBCs to bind to the endothelium [32]. This can predispose to the formation of microaggregates and the occlusion of small blood vessels [33]. Finally, the exposure of PS is associated with the formation of microvesicles [28], which are thought to mediate many of the important adverse effects associated with RBC transfusions.

Vesiculation

Microvesicles (MVs) are small phospholipid-bound particles released by a variety of cell types. They consist of cytosolic contents surrounded by a bilayer membrane expressing surface markers of their parent cells. While the release of MVs serves important physiologic roles in vivo, the generation of RBC-derived vesicles is greatly enhanced by changes that occur during storage [34]. The most significant of these are the oxidation of membrane lipids and cytoskeletal proteins, which weaken the association between the cell membrane and the underlying cytoskeleton [35], and the translocation of PS to the external phospholipid layer [28, 36]. As a result of these and other lesions, the concentration of MVs found in RCCs increases with storage duration [37].

Importantly, pathologic vesiculation of stored RBCs is thought to continue after transfusion [28]. The accumulation of lesions during storage renders these cells more susceptible to the physiological stresses, resulting in the continued production of MVs in vivo [38].

Following their release, RBC-derived MVs are rapidly cleared from circulation by splenic and hepatic phagocytes [37]. Despite their short half-life, transfused MVs are believed to have a variety of important effects. Specifically, they have been shown to promote coagulation, impair hypoxic vasodilation, and amplify the immunomodulatory effects of transfusions.

RBC-derived MVs have been shown *in vitro* and in animal models to induce a hypercoagulable state with dose-dependent increases in thrombin and fibrinogen activation [39]. In humans, MV formation is known to be increased in prothrombotic disease states such as sickle cell disease and hemolytic anemia [40]. Several mechanisms have been proposed to account for these observations. The exposure of PS on MVs is thought to enhance thrombin formation by promoting the assembly of coagulation complexes [33]. It has also been shown that RBC-derived MVs are capable of initiating the coagulation cascade in the absence of tissue factor, mostly likely via the factor XII-dependent intrinsic pathway [41].

RBC-derived MVs also act as potent scavengers of nitric oxide (NO). Hemoglobin reacts readily with NO to generate the biologically inactive products, nitrate and methemoglobin [42]. Under physiological conditions, the rate at which NO is consumed through this reaction is limited by the compartmentalization of hemoglobin within RBCs. Hemoglobin contained within MVs reacts with NO at approximately 1000 times this rate [37]. As a result, RBC-derived MVs have the potential to significantly limit the bioavailability of NO and disrupt vascular homeostasis.

Finally, RBC-derived MVs are thought to contribute to the immunomodulatory effects of transfusions [43]. While their clinical significance is debated, RBC-derived MVs have been shown to have a variety of both immunosuppressive and proinflammatory effects. They can suppress macrophage activity, inhibiting the release of the proinflammatory mediators TNF-α and IL-8 [44]. They have also been implicated in the pathogenesis of transfusion-associated acute lung injury (TRALI) through the activation of neutrophils [45] and fixation of complement [46].

Decreased Deformability

During preservation and storage, RBCs undergo a predictable sequence of morphological changes [47]. Normal RBCs are characterized by a highly flexible cytoskeleton and a biconcave, discoid morphology. With the accumulation of biochemical lesions during storage, RBCs gradually adopt a spherical form [16]. The proportion of RBCs demonstrating this abnormality rises steadily with the duration of storage [47].

The etiology of these morphological changes is complex, but likely involves dysfunction in cytoskeletal-membrane interaction as well as an increase in cytosolic viscosity. The oxidation of transmembrane and cytoskeletal proteins weakens the cytoskeleton and causes a decrease in the flexibility of the cellular envelope [33]. Likewise, the degree of lipid peroxidation has been shown to correlate directly with the rigidity of the cell membrane [32]. Impaired phosphorylation of cytoskeletal proteins, secondary to the depletion of ATP, may also contribute to a loss of flexibility [48].

In vivo, fluctuation in the levels of non-hemoglobin-bound 2,3-DPG serves to modulate the rigidity of the cytoskeleton [49]. The depletion of 2,3-DPG during storage disrupts this regulatory mechanism and is thought to further impair the deformability of transfused RBCs.

The loss of cell surface area associated with the increase in membrane vesiculation during storage may also contribute to the decrease in RBC deformability. The resulting rise in cell density and hemoglobin concentration is associated with greater cytosolic viscosity [50, 51]. These changes in the mechanical properties of RBCs have significant effects on the flow of transfused cells through the vasculature. The loss of flexibility impairs the ability of stored RBCs to traverse small vessels [33]. It also forces these cells into the normally cell-free layer adjacent to the vessel wall, where they scavenge endothelium-derived NO and induce vasoconstriction [52]. The structural changes that occur during storage are also thought to contribute to the significant rate of extravascular hemolysis following transfusion. Decreased deformability results in slower transit of transfused RBCs through the liver and spleen. This increases the time that resident macrophages can interact with these RBCs and thus increases the chance of phagocytosis [53].

Physiological Considerations

While RBC transfusions are potentially lifesaving interventions, they are also associated with a variety of adverse outcomes. These are only partially attributable to rare events such as hemolytic transfusion reactions, transfusion-transmitted infections, TRALI, or transfusion-associated circulatory overload. They are also the result of the predictable changes occurring in RBCs during processing and storage.

Hemolysis

While rates of hemolysis during storage are low, averaging 0.3–0.4% at 6 weeks, up to a quarter of stored RBCs may be cleared from circulation within hours of a transfusion [54]. The clearance of transfused RBCs from circulation lowers hematocrit and thus attenuates the intended benefits of transfusion. More significant, however, are the adverse effects associated with a supraphysiologic rate of hemolysis. Extravascular hemolysis following transfusion of stored RBCs can easily exceed the physiological capacity to sequester iron, resulting in the release of non-transferrin bound iron into circulation [55]. Iron is a limiting nutrient for many bacteria and thus the presence of free iron increases the risk of infection and sepsis [56]. Non-transferrin bound iron may

also cause oxidative injury through the production of reactive oxygen species via the Fenton reaction [16]. These effects are amplified, predictably, with the transfusion of multiple RCC units.

Oxygen Delivery

The delivery of oxygen to tissues is primarily a function of blood oxygen content and blood flow. RBCs are understood to be important determinants of both. While transfusions are generally intended to improve oxygen delivery, storage lesions compromise the ability of transfused RBCs to effectively transport oxygen to tissues.

As previously described, the oxidation of hemoglobin to methemoglobin renders the complex unable to transport oxygen [24]. While methemoglobin may be reduced to its functional form, prolonged exposure to oxidative stress can result in the irreversible degradation of hemoglobin [19]. In either case, the capacity of RBCs to carry oxygen is reduced.

The depletion of 2,3-DPG and ATP during storage also causes an increase in the affinity of hemoglobin for oxygen. The resulting leftward shift of the oxygen-dissociation curve impairs offloading of oxygen and thus compromises the ability of tissue to take up oxygen. Following transfusion, intracellular stores of 2,3-DPG are regenerated quickly, with a greater than 50% recovery within 7 hours, and full recovery to pre-transfusion levels within 48-72 hours [57]. In the immediate post-transfusion period, however, the characteristics of transfused RBCs remain significantly altered.

RBCs are also key mediators of hypoxic vasodilation, modulating the availability of nitric oxide in response to local oxygen levels [58]. As such, RBCs play an essential role in regulating blood flow to tissues and the matching of oxygen supply to metabolic demand. The transfusion of stored RBCs may induce vascular dysfunction by disrupting NO signaling [59]. Cell-free hemoglobin, whether contained within MVs or free in solution, reacts readily with NO [42]. As previously described, there is also increased NO scavenging by stored RBCs themselves due to changes in their rheologic properties [59]. In each case, the transfusion of RBCs impairs NO-mediated hypoxic vasodilation, disrupting an essential regulatory mechanism for maintaining oxygen homeostasis.

Hemostasis and Thrombosis

RBCs are recognized to play an important role in both hemostasis and thrombosis. Many of the changes that occur during storage serve to amplify the procoagulant effects of RBCs. The significance of this phenomenon depends upon the clinical context. In the setting of acute hemorrhage, for example, the procoagulant effects of transfusion may aid in hemostasis [33]. In other settings, however, transfusions are associated with an increased risk of thrombosis and thromboembolism (VTE) [60].

The strong procoagulant effects of transfusion are primarily mediated by the structural and biochemical alteration of RBCs during storage. Among these changes is the exposure of PS on the surfaces of transfused RBCs, which enhances the formation of thrombin. The externalization of PS also promotes adhesion to the endothelium, which can lead to the formation of RBC aggregates and occlusion of small blood vessels [32]. The transfusion of PS-enriched MVs has similar effects.

The impaired deformability of long-stored RBCs further contributes to their thrombogenic potential. Normally, RBCs depend on a flexible cytoskeleton and membrane to traverse the microvasculature. Rigid cells can obstruct these vessels and prevent the delivery of oxygen to more distal tissues [33].

In addition to inducing vasodilation, NO is a potent inhibitor of platelet aggregation and endothelial cell activation. By reducing the bioavailability of NO in the vasculature, the transfusion of stored RBCs promotes platelet activation [61]. Finally, the increase in hematocrit associated with RBC transfusion may contribute to its procoagulant effects. RBCs are important determinants of blood viscosity, which increases non-linearly with a higher concentration. Increased viscosity slows blood flow and promotes platelet margination, both of which contribute to clot formation [33].

Immunomodulation

Stored RBCs are known have a variety of important immunologic effects in transfused patients. This phenomenon was first noted in the 1970s with studies showing an association between RBC transfusion and improved graft survival following organ transplantation [62–64]. In most contexts, however, the immunosuppressive effects of transfusion have been linked to adverse clinical outcomes. For example, studies have shown a dose-dependent correlation between RBC transfusion and rates of nosocomial infection in critically ill patients [65]. Transfusion has also been associated with an increased risk of cancer recurrence following surgical resection [66]. There is also mounting evidence that RBC transfusion can have proinflammatory effects [67]. While the clinical implications of these findings are yet unclear, multiple preclinical studies have shown that stored RBCs can trigger the release of pro-inflammatory cytokines and promote the activation of both neutrophils and monocytes [43].

Leukoreduction has been shown to greatly reduce the immunomodulatory effects of RBC transfusion, suggesting that WBCs play a significant role in mediating these effects [43]. Various RBC-derived factors have also been implicated in transfusion-related immunomodulation. Transfusions deliver a significant iron load, for example, which has both proinflammatory and immunosuppressive effects [43]. Finally, RBC-derived MVs have significant

proinflammatory activity and may play a role in the pathogenesis of TRALI [9].

Clinical Outcomes

Despite the accumulation of metabolic and structural lesions in RBCs during storage, recent studies have failed to show a significant difference in clinical outcomes based upon the age of the transfused blood unit [68]. These findings remain controversial, however, and it has been speculated that variability in processing methods and donor characteristics could obscure the effect of storage time on clinical outcomes [69].

Component Therapy: All Things "Yellow"

Historically, component therapy involving a transfusion was employed to resolve disruptions of hemostasis that involved dysfunctional or low values of plasma proteins, disruption of the vascular endothelium, the complement system and immunoglobulins. Blood may be transfused as whole blood or as one of its components. Because patients seldom require all of the components of whole blood, it may seem intuitive to transfuse only that portion needed by the patient for a specific condition or disease. This treatment, known as "blood component therapy", allows several patients to benefit from one unit of donated whole blood. Blood components without red blood cells is referred to as plasma [70]. Blood plasma is a yellow liquid component of blood that holds the blood cells of whole blood in suspension. It is the liquid part of the blood that carries cells and proteins throughout the body. It makes up about 55% of the body's total blood volume. It is the intravascular fluid part of extracellular fluid (all body fluid outside cells). It is mostly water (up to 95% by volume), and contains important dissolved proteins (6–8%) (e.g., serum albumins, globulins, and fibrinogen), glucose, clotting factors, electrolytes (Na^+, Ca^{2+}, Mg^{2+}, HCO_3^-, Cl^-, etc.), hormones, carbon dioxide (plasma being the main medium for excretory product transportation), and oxygen. It plays a vital role in an intravascular osmotic effect that keeps electrolyte concentration balanced and protects the body from infection and other blood disorders.

Component therapy has been compared to whole blood in the treatment of acute blood loss in trauma with no difference discovered between survivorship [71]. The study points out that due to complex methodology, it is more than challenging to do this comparison. Due to complex project design, no conclusion could be drawn to answer the question. Component therapy is not less suitable for transfusion when compared to whole blood transfusion in this population.

Plasma Transfusions

The history of plasma extends as far back as Vesalius (1514–1564). The discovery of fibrinogen by William Henson in 1770, led to the study plasma, as encountering a foreign surface, endothelium. Charles Drew (June 3, 1904 – April 1, 1950) was a surgeon and medical researcher in the field of blood transfusions who developed techniques for blood storage. He applied his expert knowledge to developing large-scale blood banks early in World War II [72]. Dr. José Antonio Grifols Lucas, in, 1940, pioneered a technique called plasmapheresis, where a donor's red blood cells would be returned to their body almost immediately after the separation of the blood plasma. This technique is still in practice today [73, 74].

The Origin of Plasmapheresis Leading to Fresh Frozen Plasma

Clotting factors become activated and clotting proceeds by trapping RBCs in the plasma and preventing the separation of plasma from blood. Plasma is a yellowish liquid component of blood that suspends the blood cells of whole blood. It is the liquid part of the blood that carries cells and proteins throughout the body. It makes up about 55% of the intravascular portion of the body's blood. While plasma is mostly water, it contains important dissolved proteins such as: globulins, fibrinogen, glucose, clotting factors, electrolytes, hormones, carbon dioxide, and oxygen. These total more than 500 distinct proteins in plasma. Plasma is the main medium for excretory product transportation; playing a vital role in an intravascular osmotic effect that keeps electrolyte concentration balanced and protects the body from infection and other blood disorders.

The use of blood plasma as a substitute for whole blood and for other transfusion purposes was proposed in 1918 [74]. Dried plasma, in powder or strips of material format were developed and first used in World War II. Prior to the United States' involvement in the war, only liquid plasma and whole blood were used. Plasma is separated from whole blood by centrifugation (apheresis). There are several preparations of plasma: fresh frozen plasma (FFP), plasma frozen within 24 hours (PF24), thawed plasma (TF), liquid plasma (LP), and solid detergent plasma [75].

Whole blood contains various immune cells; mononuclear (monocytes and lymphocytes) apolymorphonuclear cells (granulocytes). Density gradient centrifugation allows the separation of immune cells from other whole blood components such as red blood cells, platelets and plasma.

Plasma is FDA approved and is stored at 1–6 °C for up to 40 days. Because liquid plasma is stored in a liquid state, it

is ready for immediate administration, thus making it ideal for the transport environment. Plasma is separated from whole blood at any time from collection up to 5 days *after the whole blood unit expires*. Whole blood is typically collected in CPD or CP2D anticoagulant preservative solution with a 21 days shelf life in the U.S. Liquid Plasma has a maximum shelf life of 26 days and is stored refrigerated at 1-6 degrees. The quantity of certain coagulation factors varies within each unit of plasma. Factor V and Factor VII decline in a plasma product stored for this length of time. Adequate quantities remain to be useful as a starting point in urgent situations.

Liquid plasma is separated and prepared from whole blood in a liquid state, and does not need to be thawed prior to transfusion. So, it can be quite useful in "the initial treatment of patients who are undergoing massive transfusion" (*Circular of Information for the Use of Human Blood and Blood Components*, November 2013, page 21). Growing numbers of trauma centers are using Liquid Plasma as a "bridge" to a thawed plasma component [76].

Solvent/detergent-treated plasma (SD-plasma) reduces the risks associated with the use of untreated FFP. Solvent/detergent technology eliminates the risk of transmission of viruses, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV). The SD-plasma manufacturing process involves pooling thousands of single-donor plasma units [77]. This reduces the virus load through dilution and neutralizes antibodies. The plasma pool also lowers the antibody titers against blood cells and plasma proteins. This allows a more standardized content of plasma proteins.

PF 24 PF24 stands for "Plasma frozen within 24 hours after phlebotomy," and is also known as "FP24" [78]. In the US, this product can be made from either whole blood or apheresis collection (note the contrast to PF24's similar-sounding sibling "PF24RT24". PF24 differs from "fresh frozen plasma" (FFP) in a very specific way:

- **FFP**: In a freezer (at < −18 °C) within 8 hours of collection
- **PF24**: In a refrigerator (at 1–6 °C) within 8 hours of collection

  In a freezer (at < −18 °C) within 24 hours of collection

The 16-hour difference in freezer placement between the two products leads to mild to moderate decreases in the "labile coagulation factors" in PF24 [79]. There is some decrease in factor VIII when compared to FFP, but the levels of factor V are not significantly different between the two products. There is also a decrease in the clotting inhibitor Protein C in PF24. Theoretically, PF24 should not be used in clinical situations where a patient has a deficit of either factors V or VIII. Clinically, the two plasma products are used interchangeably. At this time, PF24 cannot be used to make cryoprecipitate (due to decreased levels of FVIII), but like FFP, it can be relabeled as Thawed Plasma [80].

All forms of plasma may be used for management of bleeding with a specific loss of factors that are essential to clot formation and clot stability. FFP and PF24 are used interchangeably. TP and LP are not indicated for specific factor loss. Products that have higher concentrations of the missing factor should be used. Solvent-detergent plasma is indicated for plasma exchange in thrombotic thrombocytopenic purpura. (TTP), and in cases of acquired multiple factor loss as in liver transplantation and cardiac surgery [80].

Transfusion with FFP is in the process of being replaced by plasma components that are rich in factors and proteins. FFP is indicated when particular factor concentrates are not available. FFP will remain relevant when there is an acquired loss of factors and endothelial dysfunction [81]. Endothelial cells impact various steps of the coagulation cascade. These cells inhibit the tissue factor cascade. Endothelial cells balance clotting and fibrinolysis [81].

Massive transfusion protocols (MTP) are meant to deliver a balanced resuscitation, avoid coagulopathies, and generally deliver 1unit of PRBCs, I unit of FFP and 1 unit of platelets in each cycle [82]. The protocol is not hemostatic and large quantities of FFP, when factor levels are borderline for clotting, may lead to transfusion related lung injury (TRALI) and circulatory overload [83]. TRALI is believed to be the result of development of alloantibodies from previous transfusions or from pregnant patient's donations. Collection only from males or never pregnant females have reduced TRALI significantly [83].

4-Factor prothrombin complex, Kcentra, [84] is ideal for complete warfarin reversal. The volume is low and mitigates TRALI and fluid overload from FFP transfusions. When Kcentra is not available, FFP is still used. For thrombotic thrombocytopenic purpura (TTP), treatment consists of an exchange transfusion to rid blood of ADAMTS-13 [85]. This enzyme prevents the formation of thrombi in small vessels that are rich in platelets resulting in thrombocytopenia and microangiopathic hemolytic anemia which essentially defines TTP. Biologics are now on the market and it remains to be determined over the efficacy of these medications.

The Covid-19 pandemic renewed the role of convalescent plasma for the clearing of viruses. Convalescent plasma is generated from donors who had already been infected by the virus. Collection, storage and transfusion follow the same technology as other plasma products. The dose for each virus is not known and there are no trials to determine efficacy. The urgency of need remains high and is helpful in patients where other therapies have failed. With the progress in medi-

cal virology, monoclonal antibodies for treatment and vaccines for prevention remain the leading science attempts to stop the Covid-19 pandemic.

Cryoprecipitate

The term "cryo" stems from the process of preparation. FFP is thawed between 1 and 6 degrees Celsius. Cold insoluble proteins are derived and are then centrifuged to allow the separation of distinct proteins [86]. The proteins include fibrinogen, factor VIII, Factor XIII, von Willebrand factor and fibronectin. It is then frozen in 10–20 cc volumes and stored for up to 12 months. Once thawed, it must be administered within 4 hours.

Cryoprecipitate was used in the 1970s-1990s for the treatment of Hemophilia A and an array of factor deficiencies. A transfusion is triggered by a fibrinogen level less than 100 mg/ml. One unit of "cryo" per 10 kg of body weight commonly raises the plasma fibrinogen concentration by approximately 50 mg/dl [87].

Initially in the 1960s, cryoprecipitate was used for the treatment of Hemophilia A and then evolved to massive transfusions in the setting of liver transplantation, hemorrhage and obstetrical complications. Fibrinogen is the earliest factor to be depleted in blood loss due to hemodilution and frank blood loss. Cryoprecipitate that has high fibrinogen levels as emerged as the first blood product administered in trauma induced coagulopathy. The loss of fibrinogen leads to fibrinolysis, low serum levels and poorly functioning fibrinogen. In a paper on combat induced bleeding, the early use of fibrinogen lead to an increase of survivorship [88].

Cryoprecipitate has long history of treating inherited coagulopathies outside of trauma. Its role in trauma induced hemorrhage is intuitive but has only recently studied, "The Role of cryoprecipitate in massively transfused patients: Results from the Trauma Quality Improvement Program data base may change your mind." With an excellent study design, it was determined that adjunctive use of cryoprecipitate reduced mortality without additive complications [89].

It has been suggested that fibrinogen concentrates can replace cryoprecipitate in most clinical scenarios. Jensen at al. [90]. compared the efficacy and safety of fibrinogen concentrate to cryoprecipitate in bleeding patients. An IRB would not approve patients being randomized between the two preparations of fibrinogen, let alone the almost impossible task of having meaningful data. To answer the question, electronic data bases of two randomized no-randomized trials were reviewed in the Central Registered Cochrane, EMBASE, and Medline and non-randomized trials (meta-analysis) were subjected to intensive scrutiny. Mortality was the final endpoint [91].

Treating hyperfibrinolyis is a dangerous clinical event, as treatment can either stop the process of generating a stable clot or generate a thromboembolic event. Aminocaproic acid or tranexamic acid prevent fibrinolysis by giving them as early as possible in bleeding states [92]. They are a double edge sword as they will prevent fibrinolysis that can leading to a thromboembolic event. Cryoprecipitate, fibrinogen concentrate and Factor XIII have been used to both prevent and treat fibrinolysis. Cushing et al. compared these therapeutics to determine if one was better than the other two with the addition of aminocaproic acid [93]. Blood specimens were "spiked with tissue plasminogen activator" (TPA) that usually prevents clot stability and promotes fibrinolysis [94]. The impact of these specimens was measured by thromboelastography, cryoprecipitate was able to mitigate hyperfibrinolysis better than fibrinogen concentrates in an in-vitro model. This suggests a significant cost reduction is a significant factor in treating hyperfibirnolysis [95].

Platelets

Platelets are non-nucleated blood components that are prepared for transfusion by variable centrifugation of whole blood. Platelets are the smallest and lightest cells components of blood and float to the top after spinning. They are prepared by three different methods: (1) Platelet rich plasma concentrates (PRP PC). (2) Buffy coat platelet concentrates (BC-PC). (3) Apheresis-platelet concentrates. The three preparations are collected by different speeds of centrifugation. The danger of this process is that platelets may become activated [96].

Platelets are non-nucleated cells that circulate in the blood stream for 7–10 days. They have three distinct granules: alpha granules, dense delta granules and lysosomes [97].

Alpha granules contain proteins, chemokines and growth factor. Delta granules contain ADP, serotonin, polyphosphates, glutamate, histamine and calcium that are vital for hemostasis. Platelet lysosomes contain enzymes that degrade glycoproteins, glycolipids, and glucosamine. The main difference between glycolipid and glycoprotein is that glycolipid is a carbohydrate-attached lipid whereas a glycoprotein is a carbohydrate-attached protein. Glycolipids and glycoproteins stabilize membranes by forming hydrogen bonds with surrounding water molecules. They are also sites where drugs, hormones and antibodies bind, which act as receptors for cell signaling [98]. When the chemical binds to the receptor it elicits a response from the cell. This may cause a direct response or set off a cascade of events inside the cell. This process is known as cell communication or cell signaling [98].

Platelets have an established role in hemostasis and thrombosis. Platelets adhere to the injured vessel wall. Adherence leads to signaling cascades that are dependent on glycoproteins on the cell surface. NO and prostacyclin maintain the integrity of blood vessel membranes and are reduced with endothelial disruption and when matrix proteins are exposed to the circulation [99].

Adhesion and activation of platelets leads to recruitment of additional platelets and from three dimensional aggregates. Additional platelets are activated and aggregate in response to platelet release of thromboxane and ADP. When blood leaks into the tissue, they are exposed to tissue factor expressed by the cells that comprise the vessel. Activated tissue factor leads the generation of thrombin which mediates further platelet aggregation. This combination causes the clot to retract to "plug the hole' where the injury to the vessel occurred and starts the generation of fibrin. The fibrin-platelet network solidifies the plug generating a network of clot stability, over activity of this process may lead to thrombosis.

The platelet plug incites leukocytes to migrate to newly formed clot. Essentially, platelets have two roles: hemostasis and immunity at the site of vessel injury. Platelet activation leads to the release of the contents of the delta granules: serotonin and RANTES (CCLL5) that are known to mediate T cell activation and differentiation [100]. Activation attracts monocytes, eosinophils, B and T cells, and natural killer cells.

Platelets other roles include interactions during inflammation include a contribution in murine models of atherosclerosis, increased thrombotic risk in cancer patients, the destruction of a particular host of invading bacteria and viruses. The mechanism of action of each of these roles is known and comprehensive. They interact with monocytes, eosinophils, B-cells, and T cells and modify the inflammation process. Platelets are activated by monocytes and their bond is considered a marker of platelet activation. Platelets decrease apoptosis in eosinophils and may enhance an allergic response. Clinically, the prolongation of eosinophilic life may lead to many disease states as in asthma [101].

It is not uncommon to transfuse platelets at the same time that a lumbar puncture for chemotherapy is performed. The same scenario exists in ITP when a spleen is intact. It is assumed that the transfused platelets are effective shortly after infusion [102, 103] A great variety of patient- and product-related factors influence the outcome of platelet transfusions. These factors are almost countless. Physical factors include weight and height, or are pathological such as splenomegaly, fever, infection, disseminated intravascular coagulation, and previous HLA alloimmunization. Platelet factors that are associated with impaired responses are giving a decreased dose of platelets, ABO incompatible products, and platelets stored for more than 48 hours. If you eliminate these factors, transfusion increases the number of platelets in your blood straight away. For some people, the benefits may only be temporary and they may need more transfusions.

Platelet Activation and Adherence

Endothelial damage leads to collagen exposure that binds circulating platelets. Specifically, platelets bind to collagen specific glycoprotein Ia/lla receptors. Adherence is strengthened by von Willebrand factor (vWF) that is released from surface receptors of the endothelium and from platelets. These interactions activate platelets. vWF forms additional links between the platelets' glycoprotein IIb/IX/V and the collagen based glycoproteins [104].

This localization of platelets to the extracellular matrix increases collagen interaction with platelet glycoprotein VI. Binding of collagen to glycoprotein VI starts a cascade resulting in activation of platelet integrins [105]. Integrins are transmembrane receptors that facilitate cell-cell and cell-extracellular matrix. They are heterodimers, *in* that they always have just two subunits: α (alpha) and β (beta) Activated integrins lead to tight binding of platelets to the extracellular matrix. This process adheres platelets to the site of injury [105].

Activated platelets release contents of stored granules into plasma. The granules include ADP, serotonin, platelet-activating factor, vWF, platelet factor 4, and thromboxane A_2 (TXA_2) Activated plates activate additional platelets. The granules' contents activate a G-linked protein receptor cascade [106]. This cascade, leads to an increase in calcium concentration in the platelets' cytosol. Calcium activates protein kinase C, which, in turn, activates phospholipase A_2 (PLA_2). PLA_2 then modifies the integrin membrane glycoprotein IIb/IIIa, increasing its affinity to bind fibrinogen [107]. Activated platelets change shape from spherical to stellate, and the fibrinogen cross-links with glycoprotein IIb/IIIa aid in aggregation of adjacent platelets [108].

Key Points

- Component Therapy is very effective in certain specific coagulation disorders.
- In cases of hemorrhage, point of care testing can guide which component is needed
- Cryoprecipitate should be first component administered in most cases of trauma
- Several units of Fresh Frozen Plasma may be needed to control a coagulation disorder at the risk of developing

circulatory overload (TACO) or transfusion related lung injury.

- Artificial factor concentrates may replace FFP and Cryoprecipitate. Adverse effects of component therapy will no longer be a factor
- Platelet function deteriorates very quickly when the integrity of the vascular endothelium is disruptive.

References

1. McCullough J. Transfusion medicine. 4th ed. Chichester; Hoboken: Wiley; 2017.
2. Simon TL, McCullough J, Snyder EL, Solheim BG, Strauss RG. Rossi's principles of transfusion medicine. 5th ed. Chichester; Hoboken: Wiley; 2016.
3. Hess JR. An update on solutions for red cell storage. Vox Sang. 2006;91(1):13–9. PubMed PMID: 16756596. Epub 2006/06/08. eng.
4. Jones JM, Sapiano MRP, Savinkina AA, Haass KA, Baker ML, Henry RA, et al. Slowing decline in blood collection and transfusion in the United States – 2017. Transfusion. 2020;60(Suppl 2):S1–9. PubMed PMID: 32086817. PMCID: PMC7201859. Epub 2020/02/23. eng.
5. Moog R. Collection of red blood cell units by apheresis. Transfus Apher Sci. 2013;48(2):141–3. PubMed PMID: 23507240. Epub 2013/03/20. eng.
6. Harrison JF. Automated red cell collection--quality and value. Transfus Med. 2006;16(3):155–64. PubMed PMID: 16764593. Epub 2006/06/13. eng.
7. Flegel WA, Natanson C, Klein HG. Does prolonged storage of red blood cells cause harm? Br J Haematol. 2014;165(1):3–16. PubMed PMID: 24460532. PMCID: PMC5515544. Epub 2014/01/28. eng.
8. Nakao K, Wada T, Kamiyama T, Nakao M, Nagano K. A direct relationship between adenosine triphosphate-level and in vivo viability of erythrocytes. Nature. 1962;194:877–8. PubMed PMID: 14478201. Epub 1962/06/02. eng.
9. Hess JR. Measures of stored red blood cell quality. Vox Sang. 2014;107(1):1–9. PubMed PMID: 24446817. Epub 2014/01/23. eng.
10. Hess JR, Hill HR, Oliver CK, Lippert LE, Greenwalt TJ. Alkaline CPD and the preservation of RBC 2,3-DPG. Transfusion. 2002;42(6):747–52. PubMed PMID: 12147028. Epub 2002/07/31. eng.
11. Peck CC, Moore GL, Bolin RB. Adenine in blood preservation. Crit Rev Clin Lab Sci. 1981;13(3):173–212. PubMed PMID: 7018831. Epub 1981/01/01. eng.
12. Beutler E, Kuhl W. Volume control of erythrocytes during storage. The role of mannitol. Transfusion. 1988;28(4):353–7. PubMed PMID: 3133847. Epub 1988/07/01. eng.
13. Heaton WA, Holme S, Smith K, Brecher ME, Pineda A, AuBuchon JP, et al. Effects of 3-5 log10 pre-storage leucocyte depletion on red cell storage and metabolism. Br J Haematol. 1994;87(2):363–8. PubMed PMID: 7947280. Epub 1994/06/01. eng.
14. Antonelou MH, Tzounakas VL, Velentzas AD, Stamoulis KE, Kriebardis AG, Papassideri IS. Effects of pre-storage leukoreduction on stored red blood cells signaling: a time-course evaluation from shape to proteome. J Proteome. 2012;76 Spec No:220–38. PubMed PMID: 22796353. Epub 2012/07/17. eng.
15. Simancas-Racines D, Osorio D, Martí-Carvajal AJ, Arevalo-Rodriguez I. Leukoreduction for the prevention of adverse reactions from allogeneic blood transfusion. Cochrane Database Syst Rev. 2015;(12):CD009745. PubMed PMID: 26633306. Epub 2015/12/04. eng.
16. Yoshida T, Prudent M, D'Alessandro A. Red blood cell storage lesion: causes and potential clinical consequences. Blood Transfus. 2019;17(1):27–52. PubMed PMID: 30653459. PMCID: PMC6343598 Inc., and the company is commercialising a hypoxic RCC storage technology. MP declares that there are no conflicts of interest associated with this publication, but that he receives financial support (analytical measurements) from Hemanext for a research project on Hemanext bags. ADA is a founder of Omix Technologies Inc. and a consultant for Hemanext Inc. Epub 2019/01/18. eng.
17. Jóźwik M, Szczypka M, Gajewska J, Laskowska-Klita T. Antioxidant defence of red blood cells and plasma in stored human blood. Clin Chim Acta. 1997;267(2):129–42. PubMed PMID: 9469248. Epub 1998/02/20. eng.
18. Leonart MS, Nascimento AJ, Nonoyama K, Pelissari CB, Barretto OC. Enzymes and membrane proteins of ADSOL-preserved red blood cells. Sao Paulo Med J. 2000;118(2):41–5. PubMed PMID: 10772695. Epub 2000/04/20. eng.
19. Kanias T, Acker JP. Biopreservation of red blood cells--the struggle with hemoglobin oxidation. FEBS J. 2010;277(2):343–56. PubMed PMID: 19968714. Epub 2009/12/09. eng.
20. Sztiller M, Puchala M, Kowalczyk A, Bartosz G. The influence of ferrylhemoglobin and methemoglobin on the human erythrocyte membrane. Redox Rep. 2006;11(6):263–71. PubMed PMID: 17207308. Epub 2007/01/09. eng.
21. Deepa Devi KV, Manoj Kumar V, Arun P, Santhosh A, Padmakumaran Nair KG, Lakshmi LR, et al. Increased lipid peroxidation of erythrocytes in blood stored in polyvinyl chloride blood storage bags plasticized with di-[2-ethyl hexyl] phthalate and effect of antioxidants. Vox Sang. 1998;75(3):198–204. PubMed PMID: 9852407. Epub 1998/12/16. eng.
22. D'Alessandro A, Mirasole C, Zolla L. Haemoglobin glycation (Hb1Ac) increases during red blood cell storage: a MALDI-TOF mass-spectrometry-based investigation. Vox Sang. 2013;105(2):177–80. PubMed PMID: 23521396. Epub 2013/03/26. eng.
23. Inouye M, Hashimoto H, Mio T, Sumino K. Levels of lipid peroxidation product and glycated hemoglobin A1c in the erythrocytes of diabetic patients. Clin Chim Acta. 1998;276(2):163–72. PubMed PMID: 9764734. Epub 1998/10/09. eng.
24. Wright RO, Lewander WJ, Woolf AD. Methemoglobinemia: etiology, pharmacology, and clinical management. Ann Emerg Med. 1999;34(5):646–56. PubMed PMID: 10533013. Epub 1999/10/26. eng.
25. Op den Kamp JA. Lipid asymmetry in membranes. Annu Rev Biochem. 1979;48:47–71. PubMed PMID: 382989. Epub 1979/01/01. eng.
26. Seigneuret M, Devaux PF. ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. Proc Natl Acad Sci U S A. 1984;81(12):3751–5. PubMed PMID: 6587389. PMCID: PMC345297. Epub 1984/06/01. eng.
27. Verhoeven AJ, Hilarius PM, Dekkers DW, Lagerberg JW, de Korte D. Prolonged storage of red blood cells affects aminophospholipid translocase activity. Vox Sang. 2006;91(3):244–51. PubMed PMID: 16958837. Epub 2006/09/09. eng.
28. Burger P, Kostova E, Bloem E, Hilarius-Stokman P, Meijer AB, van den Berg TK, et al. Potassium leakage primes stored erythrocytes for phosphatidylserine exposure and shedding of procoagulant vesicles. Br J Haematol. 2013;160(3):377–86. PubMed PMID: 23190498. Epub 2012/11/30. eng.

29. Kay JG, Grinstein S. Phosphatidylserine-mediated cellular signaling. Adv Exp Med Biol. 2013;991:177–93. PubMed PMID: 23775696. Epub 2013/06/19. eng.
30. Boas FE, Forman L, Beutler E. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. Proc Natl Acad Sci U S A. 1998;95(6):3077–81. PubMed PMID: 9501218. PMCID: PMC19697. Epub 1998/04/18. eng.
31. Whelihan MF, Mann KG. The role of the red cell membrane in thrombin generation. Thromb Res. 2013;131(5):377–82. PubMed PMID: 23402970. Epub 2013/02/14. eng.
32. Wautier MP, Héron E, Picot J, Colin Y, Hermine O, Wautier JL. Red blood cell phosphatidylserine exposure is responsible for increased erythrocyte adhesion to endothelium in central retinal vein occlusion. J Thromb Haemost. 2011;9(5):1049–55. PubMed PMID: 21362128. Epub 2011/03/03. eng.
33. Weisel JW, Litvinov RI. Red blood cells: the forgotten player in hemostasis and thrombosis. J Thromb Haemost. 2019;17(2):271–82. PubMed PMID: doi:10.1111/jth.14360.
34. Leal JKF, Adjobo-Hermans MJW, Bosman G. Red blood cell homeostasis: mechanisms and effects of microvesicle generation in health and disease. Front Physiol. 2018;9:703. PubMed PMID: 29937736. PMCID: PMC6002509. Epub 2018/06/26. eng.
35. Sut C, Tariket S, Chou ML, Garraud O, Laradi S, Hamzeh-Cognasse H, et al. Duration of red blood cell storage and inflammatory marker generation. Blood Transfus. 2017;15(2):145–52. PubMed PMID: 28263172. PMCID: PMC5336336. Epub 2017/03/07. eng.
36. Morel O, Jesel L, Freyssinet JM, Toti F. Cellular mechanisms underlying the formation of circulating microparticles. Arterioscler Thromb Vasc Biol. 2011;31(1):15–26. PubMed PMID: 21160064. Epub 2010/12/17. eng.
37. Donadee C, Raat NJ, Kanias T, Tejero J, Lee JS, Kelley EE, et al. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. Circulation. 2011;124(4):465–76. PubMed PMID: 21747051. PMCID: PMC3891836. Epub 2011/07/13. eng.
38. Antonelou MH, Seghatchian J. Update on extracellular vesicles inside red blood cell storage units: adjust the sails closer to the new wind. Transfus Apher Sci. 2016;55(1):92–104. PubMed PMID: 27452642. Epub 2016/07/28. eng.
39. Kim Y, Xia BT, Jung AD, Chang AL, Abplanalp WA, Caldwell CC, et al. Microparticles from stored red blood cells promote a hypercoagulable state in a murine model of transfusion. Surgery. 2018;163(2):423–9. PubMed PMID: 29198748. PMCID: PMC5780240. Epub 2017/12/05. eng.
40. van Beers EJ, Schaap MC, Berckmans RJ, Nieuwland R, Sturk A, van Doormaal FF, et al. Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. Haematologica. 2009;94(11):1513–9. PubMed PMID: 19815831. PMCID: PMC2770961. Epub 2009/10/10. eng.
41. Van Der Meijden PE, Van Schilfgaarde M, Van Oerle R, Renné T, ten Cate H, Spronk HM. Platelet- and erythrocyte-derived microparticles trigger thrombin generation via factor XIIa. J Thromb Haemost. 2012;10(7):1355–62. PubMed PMID: 22537188. Epub 2012/04/28. eng.
42. Azarov I, Liu C, Reynolds H, Tsekouras Z, Lee JS, Gladwin MT, et al. Mechanisms of slower nitric oxide uptake by red blood cells and other hemoglobin-containing vesicles. J Biol Chem. 2011;286(38):33567–79. PubMed PMID: 21808057. PMCID: PMC3190873. Epub 2011/08/03. eng.
43. Remy KE, Hall MW, Cholette J, Juffermans NP, Nicol K, Doctor A, et al. Mechanisms of red blood cell transfusion-related immunomodulation. Transfusion. 2018;58(3):804–15. PubMed PMID: 29383722. PMCID: PMC6592041. Epub 2018/02/01. eng.
44. Sadallah S, Eken C, Schifferli JA. Erythrocyte-derived ectosomes have immunosuppressive properties. J Leukoc Biol. 2008;84(5):1316–25. PubMed PMID: 18685086. Epub 2008/08/08. eng.
45. Belizaire RM, Prakash PS, Richter JR, Robinson BR, Edwards MJ, Caldwell CC, et al. Microparticles from stored red blood cells activate neutrophils and cause lung injury after hemorrhage and resuscitation. J Am Coll Surg. 2012;214(4):648–55; discussion 56–7. PubMed PMID: 22342784. PMCID: PMC4034387. Epub 2012/02/22. eng.
46. Zecher D, Cumpelik A, Schifferli JA. Erythrocyte-derived microvesicles amplify systemic inflammation by thrombin-dependent activation of complement. Arterioscler Thromb Vasc Biol. 2014;34(2):313–20. PubMed PMID: 24311376. Epub 2013/12/07. eng.
47. Berezina TL, Zaets SB, Morgan C, Spillert CR, Kamiyama M, Spolarics Z, et al. Influence of storage on red blood cell rheological properties. J Surg Res. 2002;102(1):6–12. PubMed PMID: 11792145. Epub 2002/01/17. eng.
48. Park Y, Best CA, Auth T, Gov NS, Safran SA, Popescu G, et al. Metabolic remodeling of the human red blood cell membrane. Proc Natl Acad Sci U S A. 2010;107(4):1289–94. PubMed PMID: 20080583. PMCID: PMC2802590. Epub 2010/01/19. eng.
49. De Rosa MC, Carelli Alinovi C, Galtieri A, Scatena R, Giardina B. The plasma membrane of erythrocytes plays a fundamental role in the transport of oxygen, carbon dioxide and nitric oxide and in the maintenance of the reduced state of the heme iron. Gene. 2007;398(1–2):162–71. PubMed PMID: 17573207. Epub 2007/06/19. eng.
50. Bosman GJ, Werre JM, Willekens FL, Novotný VM. Erythrocyte ageing in vivo and in vitro: structural aspects and implications for transfusion. Transfus Med. 2008;18(6):335–47. PubMed PMID: 19140816. Epub 2009/01/15. eng.
51. Antonelou MH, Kriebardis AG, Papassideri IS. Aging and death signalling in mature red cells: from basic science to transfusion practice. Blood Transfus. 2010;8(Suppl 3):s39–47. PubMed PMID: 20606748. PMCID: PMC2897187. Epub 2010/07/08. eng.
52. Yalcin O, Ortiz D, Tsai AG, Johnson PC, Cabrales P. Microhemodynamic aberrations created by transfusion of stored blood. Transfusion. 2014;54(4):1015–27. PubMed PMID: 23901933. PMCID: PMC4105299. Epub 2013/08/02. eng.
53. Qadri SM, Bissinger R, Solh Z, Oldenborg PA. Eryptosis in health and disease: a paradigm shift towards understanding the (patho) physiological implications of programmed cell death of erythrocytes. Blood Rev. 2017;31(6):349–61. PubMed PMID: 28669393. Epub 2017/07/04. eng.
54. Dumont LJ, AuBuchon JP. Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials. Transfusion. 2008;48(6):1053–60. PubMed PMID: 18298603. Epub 2008/02/27. eng.
55. Rapido F, Brittenham GM, Bandyopadhyay S, La Carpia F, L'Acqua C, McMahon DJ, et al. Prolonged red cell storage before transfusion increases extravascular hemolysis. J Clin Invest. 2017;127(1):375–82. PubMed PMID: 27941245. PMCID: PMC5199711. Epub 2016/12/13. eng.
56. Hod EA, Spitalnik SL. Stored red blood cell transfusions: iron, inflammation, immunity, and infection. Transfus Clin Biol. 2012;19(3):84–9. PubMed PMID: 22682673. PMCID: PMC6551602. Epub 2012/06/12. eng.
57. Heaton A, Keegan T, Holme S. In vivo regeneration of red cell 2,3-diphosphoglycerate following transfusion of DPG-depleted AS-1, AS-3 and CPDA-1 red cells. Br J Haematol. 1989;71(1):131–6. PubMed PMID: 2492818. Epub 1989/01/01. eng.
58. Singel DJ, Stamler JS. Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin. Annu Rev Physiol. 2005;67:99–145. PubMed PMID: 15709954. Epub 2005/02/16. eng.

59. Liu C, Liu X, Janes J, Stapley R, Patel RP, Gladwin MT, et al. Mechanism of faster NO scavenging by older stored red blood cells. Redox Biol. 2014;2:211–9. PubMed PMID: 24494195. PMCID: PMC3909782. Epub 2014/02/05. eng.
60. Goel R, Patel EU, Cushing MM, Frank SM, Ness PM, Takemoto CM, et al. Association of perioperative red blood cell transfusions with Venous thromboembolism in a North American registry. JAMA Surg. 2018;153(9):826–33. PubMed PMID: 29898202. PMCID: PMC6233649. Epub 2018/06/14. eng.
61. Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. JAMA. 2005;293(13):1653–62. PubMed PMID: 15811985. Epub 2005/04/07. eng.63.
62. Opelz G, Terasaki PI. Improvement of kidney-graft survival with increased numbers of blood transfusions. N Engl J Med. 1978;299(15):799–803. PubMed PMID: 357971. Epub 1978/10/12. eng.
63. Fernández FG, Jaramillo A, Ewald G, Rogers J, Pasque MK, Mohanakumar T, et al. Blood transfusions decrease the incidence of acute rejection in cardiac allograft recipients. J Heart Lung Transplant. 2005;24(7 Suppl):S255–61. PubMed PMID: 15993782. Epub 2005/07/05. eng.
64. Opelz G, Vanrenterghem Y, Kirste G, Gray DW, Horsburgh T, Lachance JG, et al. Prospective evaluation of pretransplant blood transfusions in cadaver kidney recipients. Transplantation. 1997;63(7):964–7. PubMed PMID: 9112348. Epub 1997/04/15. eng.
65. Taylor RW, Manganaro L, O'Brien J, Trottier SJ, Parkar N, Veremakis C. Impact of allogenic packed red blood cell transfusion on nosocomial infection rates in the critically ill patient. Crit Care Med. 2002;30(10):2249–54. PubMed PMID: 12394952. Epub 2002/10/24. eng.
66. Cata JP, Wang H, Gottumukkala V, Reuben J, Sessler DI. Inflammatory response, immunosuppression, and cancer recurrence after perioperative blood transfusions. Br J Anaesth. 2013;110(5):690–701. PubMed PMID: 23599512. PMCID: PMC3630286. Epub 2013/04/20. eng.
67. Muszynski JA, Spinella PC, Cholette JM, Acker JP, Hall MW, Juffermans NP, et al. Transfusion-related immunomodulation: review of the literature and implications for pediatric critical illness. Transfusion. 2017;57(1):195–206. PubMed PMID: 27696473. Epub 2016/10/04. eng.
68. Shah A, McKechnie S, Brunskill SJ, Stanworth SJ. Fresh versus old red cell transfusions: what have the recent clinical trials found? Curr Opin Hematol. 2016;23(6):550–6. PubMed PMID: 27518928. Epub 2016/10/18. eng.
69. Sparrow RL. Red blood cell components: time to revisit the sources of variability. Blood Transfus. 2017;15(2):116–25. PubMed PMID: 28263168. PMCID: PMC5336332. Epub 2017/03/07. eng.
70. Maton A, Hopkins G, McGaughlin Johnson S, Warner J, Lahart D, Wright J. Human biology and health. Englewood Cliffs: Prentice Hall; 2017. ISBN 0-13-981176-
71. Avery P, Morton S, Tucker H, Dean L, Ross Davenport R. Whole blood transfusion versus component therapy in adult trauma patients with acute major hemorrhage. Emerg Med J. 2020;37(6):370–8.
72. Parker-Kelly D, Hobbs CP. Keeping Dr. Richards Drew's legacy alive. J Med Libr Assoc. 2019;107(3):449–53.
73. Grífols-Lucas JA. Use of plasmapheresis in blood donors. Br Med J. 1952;1(4763):854. https://doi.org/10.1136/bmj.1.4763.854. PMC 2023259. PMID 14916171.
74. Lewisohn R. The development of the technique of blood transfusion since 1907; with special reference to contributions by members of the staff of the Mount Sinai Hospital. J Mt Sinai Hosp. 1944;10:605–22.
75. Garraud O. Are all therapeutic plasma preparations the same: is it worth assessing them in clinical trials? Transfus Apher Sci. 2017;56(6):920–3.
76. Hartwell G, Barbeau J, Wade C, Holcomb J. Better hemostatic profiles of never-frozen liquid plasma compared with thawed fresh frozen plasma 1. J Trauma Acute Care Surg. 2013;74(1):84–90.
77. Marietta M, Franchini M, Bindi L, et al. Is solvent/detergent plasma better than standard fresh-frozen plasma? A systematic review and an expert consensus document. Blood Transfus. 2016;14(4):277–86.
78. Alhumaidan H, Cheves T, Holme S, Sweeney J. Stability of coagulation factors in plasma prepared after a 24-hour room temperature hold. Transfusion. 2010;50(9):1934–42.
79. Naghadeh HT, Roudkenar MH. A study of the quantity of some stable and labile coagulation factors in fresh-frozen plasma produced from whole blood stored for 24 hours in Iran. Blood Transfus. 2009;7(1):39–42.
80. Prajeeda M, Rendo MJ, Reddoch-Cardenas KM, Burris JK, Meledeo, Cap AP. Recent advances in the use of fresh frozen plasma, cryoprecipitate, immunoglobulins and clotting factors for transfusion support in patients with hematologic disease. Semin Hematol. 2020;57:73–82.
81. Rockey DC. Endothelial dysfunction in advanced liver disease. Am J Med Sci. 2015;349(1):6–16.
82. Menseses E, Bonva D, Mclenney M, Elkbulia A. Massive transfusion protocol in adult trauma population. Am J Emerg Med. 2020;38(12):2661–5.
83. Roubinian N. TACO and TRALI: biology, risk factors, and prevention strategies. Hematology Am Soc Hematol Educ Program. 2018;2018(1):585–94.
84. Schwebach AA, Waybright RA, Johnson TJ. Fixed-dose four-factor prothrombin complex concentrate for vitamin K antagonist reversal: does one dose fit all? Pharmacotherapy. 2019;39(5):599–608. https://doi.org/10.1002/phar.2261. Epub 2019 Apr 21. PMID: 30892733 Review.
85. Plautz WE, Raval JS, Dyer MR, Rollins-Raval MA, Zuckerbraun BS, Neal MD. ADAMTS13: origins, applications, and prospects. Transfusion. 2018;58(10):2453–62. https://doi.org/10.1111/trf.14804. Epub 2018 Sep 12. PMID: 30208220 87. Burka ER, Puffer T, Martinez J. Transfusion.
86. Burka ET, Puffer T, Martinez. The influence of donor characteristics and preparation methods on the potency of human cryoprecipitate. Transfusion. 1975;15(4):323–8. https://doi.org/10.1046/j.1537-2995.1975.15476034551.x.
87. Subramaniyan R, Marwaha N, Jain A, Ahluwalia J. Factors affecting the quality of cryoprecipitate. Transfus Sci. 2017;11(1):33–9. https://doi.org/10.4103/0973-6247.200778. PMID: 28316438
88. Gurney JM, Spinella PC. Blood transfusion management in the severely bleeding military patient. Curr Opin Anaesthesiol. 2018;31(2):207–14.
89. Ditillo M, Hanna K, Castanon L, Zeeshn M. The role of cryoprecipitate in massively transfused patients: improvement program database may change your mind. J Trauma Acute Care Surg. 2020;89(2):336–43.
90. Jensen NHL, Stensballe, Afshari A. Comparing efficacy and safety of fibrinogen concentrate to cryoprecipitate in bleeding patients: a systematic review. Acta Anaesthesilogica. 2016;60:1033–42.
91. Quilten ZK, Bailey M, Cameron PA, Stanworth SJ, Venardos K, Wood EM, Cooper DJ. Fibrinogen concentration and use of fibrinogen supplementation with cryoprecipitate in patients with critical bleeding receiving massive transfusion: a bi-national cohort study. Br J Haematol. 2017;179(1):131–41.
92. McCormack P. Tranexamic acid: a review of its use in the treatment of hyperfibrinolysis. Transfusion. 2012;72(5):585–617.

93. Cushing MM, Fitzgerald MM, Harris RM, Asmis LM, Haas T. Influence of cryoprecipitate, factor XIII, and fibrinogen concentrate on hyperfibrinolysis. Transfusion. 2017;57(10):2502–10. https://doi.org/10.1111/trf.14259. Epub 2017 Jul 21.
94. Gall L, Brohl K, Davenport R. Diagnosis and treatment of hyperfibrinolysis in trauma (a European perspective). Semin Thromb Hemost. 2017;43(2):224–3.
95. Levy JH, Koster A, Quinones QJ, Milling TJ, Key NS. Antifibrinolytic therapy and perioperative considerations. Anesthesiology. 2018;128(3):657.
96. Greening DW, Sparrow RL, Simpson RJ. Preparation of platelet concentrates. Methods Mol Biol (Clifton, NJ). 2011;728:267–712.
97. Ambrosio AL, Di Pietro SM. Blood mechanism of platelet alpha-granule biogenesis: study of cargo transport and the VPS33B-VPS16B complex in a model system Ambrosio AL, Di Pietro SM. Blood Adv. 2019;3(17):2617–26.
98. Koupenovaa M, Clancy L, Corkery HA, Freedman JE. Circulating platelets as mediators of immunity, inflammation and thrombosis. Circ Res. 2018;122(2):337–51.
99. Gromotowicz-Poplawawska A, Kloza AM, et al. Nitric oxide as a modulator in platelet- and endothelium-dependent antithrombotic effect of eplerenone in diabetic rats. J Physiol Pharmacol. 2019;70(2)
100. Bajogiannis C, Sachse M, Stamatelopoulos K, Stellos K. Platelet-derived chemokines in inflammation and atherosclerosis. Cytokine. 2019;122:154–7.
101. Page CP, Coyle AJ. The interaction between PAF, platelets and eosinophils in bronchial asthma. Eur Respir J Suppl. 1989;6:483s–487.
102. Slichter S, Davis K, Enright H, et al. Factors affecting post-transfusion platelet increments, platelet refractoriness, and platelet transfusion intervals in thrombocytopenic patients. Blood. 2005;105(10):4106–14.
103. Sebsebe L. Factors affecting posttransfusion platelet efficiency "close relationship between patient and product". Transfus Clin Biol. 2007;14(1):90–3.
104. Lepage AM, Lebouve JP, Cazenave C, et al. The αIIbβ3 integrin and GPIb-V-IX complex identify distinct stages in the maturation of CD34+cord blood cells to megakaryocytes. *Blood*. [cit. 2012-06-12].
105. Jandrot-Perrus M, Busfield S, Lagrue AH, Xiong X, Debili N, Chickering T, Le Couedic JP, Goodearl A, Dussault B, Fraser C, Vainchenker W, Villeval JL. Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. Blood. 2000;96(5):177–8.
106. Hattul SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb. IIIa complex during platelet activation. J Biol Chem. 1985;260(20):11107−14.
107. Calvete JJ. On the structure and function of platelet integrin alpha IIb beta 3, the fibrinogen receptor. Proc Soc Exp Biol Med. 1995;208(4):346–60.
108. Dumin JA, Dickeson SK, Stricker TP, Bhattacharyya-Pakrasi M, Roby JD, Santoro SA, Parks WC. Pro-collagenase-1 (matrix metalloproteinase-1) binds the alpha (2) beta (1) integrin upon release from keratinocytes migrating on type I collagen. J Biol Chem (United States). 2001;276(31):29368–74.

Allogeneic Blood Transfusion: Complications and Side Effects

7

Matthew Hammer

Introduction

Physicians across a diverse array of specialties are frequently tasked with the decision whether or not to transfuse patients, both in the inpatient and outpatient settings. Examples of clinical scenarios in which transfusions may be indicated are seemingly limitless and include trauma, post-partum hemorrhage, replacement of intraoperative blood loss, patients with oncologic diagnoses undergoing chemotherapy, patients with chronic anemia, etc. The physician who elects to transfuse a patient should not make that decision without weighing the benefits and often very serious risks that are inherent to blood product administration. This chapter will identify and describe the most common and most dangerous acute adverse events and side effects of blood product administration, including infectious, immunologic, and circulatory complications. Reactions that are quite rare or that tend to be clinically insignificant will not be discussed.

Infectious Complications

In past decades, transmission of viruses via allogeneic blood transfusion was lamentably a frequent event that could inflict significant morbidity and mortality on the recipient. The advent of highly sensitive screening tests significantly decreased the rate of viral transmissions, but the clinician should still be aware of this now rare but potentially devastating complication.

Human Immunodeficiency Virus

Transmission of the human immunodeficiency virus (HIV) via blood transfusion has been a long-standing concern, dating back to the outbreak of the disease in the 1980s. Since the introduction of the highly sensitive minipool nucleic acid testing in the United States in 1999, the risk of HIV transmission has markedly decreased [1]. Over a 10 year period from 1999 to 2008, the American Red Cross observed the incidence of HIV transmission via blood transfusion to be 1 in 2,000,000 donations [1]. This now exceedingly rare complication can still occur if a newly infected person donates blood in the short window period before the assay can detect the presence of the virus.

Hepatitis C Virus

Screening of donated units for the hepatitis C virus using nucleic acid testing was also implemented in the United States in 1999, marking a significant decrease in transfusion-mediated transmission of the virus, which had been fairly common prior to the introduction of this highly sensitive test [2]. Drawing on data from the American Red Cross, the risk of transmission of the hepatitis C virus occurs in less than 1 in 1,000,000 donations [2].

Hepatitis B Virus

Similarly, increasingly sensitive assays, as well as the presence of a vaccine, have led to a decreased incidence of hepatitis B transmission due to blood transfusion in the past four decades, though it still occurs more frequently than HIV and hepatitis C, possibly owing to a seroconversion window and donors with exquisitely low viral loads [3]. Estimates place the risk between 1 in 500,000 and 1,100,000 [3].

Septic Transfusion Reactions

Clinicians who administer platelets must be vigilant for septic transfusion reactions, a potentially fatal complication.

M. Hammer (✉)
Department of Anesthesiology, Mayo Clinic Arizona, Phoenix, AZ, USA
e-mail: hammer.matthew@mayo.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_7

Because platelets are stored at room temperature, they are susceptible to bacterial contamination, a problem that has persisted despite multiple advancements in surveillance and testing [4]. Incidence in platelet transfusions remains approximately 1 in 5000 [5]. Patients typically present with signs of the systemic inflammatory response syndrome. If septic transfusion reaction is suspected, the clinician should promptly order Gram stain and culture of all transfused units [5].

Immune-Mediated Complications

Whereas the incidence of transmission of the aforementioned viral pathogens via transfusion of blood products has become an extremely rare event, the clinician must be aware of the risks of immunologic complications, which are far more common. This broad term is divided into several different subtypes, with varying degrees of morbidity and mortality.

Hemolytic Transfusion Reaction

Hemolytic transfusion reactions, characterized by the destruction of transfused red blood cells in patients with antibodies, are among the most emergent and dangerous clinical scenarios in the setting of blood transfusions. These reactions are further subdivided into acute (onset of signs and symptoms within 24 hours of product administration) and delayed (onset of signs and symptoms after 24 hours of product administration). While significantly more common in previous decades, the incidence of fatal hemolytic transfusion reactions has fallen significantly since 2005, with one to four annual deaths in recent years [6]. This has coincided with a decrease in transfusion of ABO-incompatible blood due to improved safety protocols and electronic verification prior to transfusion. A 2016 practice guideline form the American Association of Blood Banks estimated the risk of fatal hemolytic transfusion reaction at 1 in 972,000 units [7]. The Food and Drug Administration maintains a database for all transfusion related deaths, and from 2014 to 2018, hemolytic reactions were implicated in 18% of deaths – 7% ABO mediated, and 11% for non-ABO mediated (FDA). The classically reported triad of fever, flank pain, and red urine should alert the clinician to a possible acute hemolytic transfusion reaction, but other symptoms such as rigors, chills, hypotension, and oliguria may also be present [6]. If a reaction is suspected, the transfusion should be stopped and the unit returned to the blood bank for further ABO, Rh factor, and antibody testing. A direct antiglobulin (Coombs') test confirms the presence of hemolysis, but false negative results can occur if the hemolyzed red blood cells have been cleared from the circulation prior to obtaining the sample [6]. The treatment for hemolytic transfusions consists of supportive care. This entails ensuring adequate hydration as well as adequate perfusion to organ systems. In severe cases, more advanced therapies such as vasopressors, inotropes, and ventilatory support may be indicated.

There is currently no evidence for any specific intervention [5], and therapies should be tailored to the clinical condition of each individual patient.

Delayed hemolytic transfusion reactions refer to hemolytic events that occur between 24 hours and 30 days of blood administration. Such reactions tend to be more mild than acute hemolytic reactions and are often clinically silent. However signs and symptoms can be more severe in patients with underlying sickle cell disease [6]. Patients most commonly present with anemia and jaundice. Initial laboratory investigation may reveal low hemoglobin, elevated lactate dehydrogenase, low haptoglobin, and elevated bilirubin. A thorough transfusion history should be conducted. Patients should receive subsequent crossmatched units as needed to treat the anemia. Further treatment is rarely indicated [6].

Allergic/Anaphylactic Complications

Allergic Transfusion Reactions

Allergic transfusion reactions are relatively common, occurring in up to 3% of transfusions [8] and possibly more due to underreporting. They are most commonly associated with urticaria, pruritis, and flushing, though these can be variable [9]. Both allergen and non-allergen dependent pathways have been postulated, with type I hypersensitivity IgE-mediated histamine release implicated in the former [10]. In most cases the signs and symptoms remain mild but they can progress to more severe symptoms (see Anaphylactic Transfusion Reactions below) If allergic transfusion is suspected, the transfusion should be temporarily halted and the patient should receive an antihistamine such as diphenhydramine. If the patient only sustained cutaneous manifestations and they resolved after treatment, the transfusion may be resumed; this is one of the rare adverse events in which continuation of the transfusion can be considered. However, if symptoms persist or worsen, the transfusion should not be resumed, and a more serious etiology for the clinical presentation should be considered. Studies have not shown pretreatment with antihistamines to be effective in preventing allergic transfusion reactions [8], though patients who have experienced past allergic transfusion reactions should be vigilantly monitored for complications in the setting of future transfusions. Retrospective studies have shown removal of plasma from red blood cells and platelets via washing or concentrating to be effective in decreasing the

incidence of allergic transfusion reactions, though it is recommended these measures only be taken in patients who have had severe reactions [11].

Anaphylactic Transfusion Reactions

At the other, more severe end of the type I hypersensitivity reaction spectrum lie anaphylactic transfusion reactions, which tend to occur within the first hour of blood product administration and are characterized by angioedema and cardiovascular and respiratory compromise [12]. These life-threatening events are rare, on the order of 8 per 100,000 units transfused [5]. Patients with suspected anaphylactic transfusion reactions should be immediately treated with epinephrine. Additional vasopressor support and the placement of an artificial airway may also be necessary. The clinician can also consider steroids and antihistamines [5]. IgA deficiency has been implicated, but the exact cause in the majority of cases remains unknown. Histamine mediated allergic reactions can develop acutely within hours of product administration. Patients with a history of an anaphylactic reaction should receive washed or concentrated units for subsequent transfusions.

Febrile Transfusion Reactions

Febrile transfusion reactions are one of the most common adverse events in the setting of transfusions. These reactions most frequently occur in with platelet and packed red blood cell administration. A febrile transfusion reaction is characterized by an increase in temperature of 1–2 °C [13]. An increase of greater than two degrees should prompt the clinician to consider more serious etiologies, such as hemolytic transfusion reaction or sepsis. Associated symptoms vary and may include chills, rigors, malaise, and hypotension. Donor white blood cells have been implicated in the pathogenesis of febrile transfusion reactions, either through interaction with recipient antibodies or through accumulation of pro-inflammatory cytokines during storage and prior to administration [14]. If a febrile transfusion reaction is suspected, the transfusion should be stopped, and the patient's hemodynamic and clinical status should be monitored closely, to rule out more severe causes of fever. Anti-pyretic medication and meperidine may be given if the patient is in discomfort or is suffering from rigors. The majority of these reactions are self-limiting, and in the absence of persistent, marked elevation of temperature or profound comorbid conditions, hospital admission is generally not warranted. A 2019 meta-analysis did not reveal a statistically significant decreased incidence in febrile transfusion reactions with pretreatment with acetaminophen and an anti-histamine, though only a very small percentage of patients in the studies had a previous history of transfusion reaction, and the benefit of pretreatment in such patients remains controversial [15]. Universal leukocyte reduction has been shown in retrospective studies to reduce the incidence of. febrile transfusion reactions in patients receiving red blood cell and platelet transfusions [16].

Cardiac and Pulmonary Complications

Transfusion Associated Circulatory Overload

Transfusion Associated Circulatory Overload (TACO) refers to an acute, life-threatening, commonly difficult to diagnose clinical scenario, often in the setting of rapid administration of multiple units of blood products. Initially, the reaction can be mischaracterized as transfusion related acute lung injury (TRALI), described below, since both frequently present with respiratory distress. Several clinical definitions have emerged since 2000, with the 2016 National Healthcare Safety Network (NHSN) among the most recent [17]. According to this classification, the patient must exhibit three or more of the following within 6 hours of blood product administration: respiratory distress, positive fluid balance, elevated brain natriuretic peptide (BNP), radiographic evidence of pulmonary edema, evidence of left heart failure, and evidence of elevated central venous pressure [17]. The pathophysiology has not been completely elucidated. A two-hit hypothesis has been proposed in which the first hit is a patient-specific comorbid condition, such as congestive heart failure or renal dysfunction, and the second hit occurs with transfusions that are excessive or too rapid for the patient's cardiovascular system to process, or a patient reaction to a blood product component [18]. These combine to cause the signs and symptoms listed above. Despite extensive research investigating multiple possible pathways that mediate TACO, supportive care remains the only available therapy.

Transfusion Associated Acute Lung Injury

Transfusion related acute lung injury is a potentially fatal pulmonary complication associated with blood product transfusions and it is a leading cause of transfusion associated mortality. Patient-related risk factors include severe liver disease, shock states, hypervolemia, chronic alcohol abuse, and elevated interleukin-8 levels [19]. A clinical diagnosis, the clinician must consider TRALI if a patient develops respiratory distress shortly after the initiation of a transfusion. Since 2004, criteria from the National Heart, Lung, and Blood Institute have been used to make the diagnosis of TRALI [20]. However, due to advances in knowl-

edge about the pathophysiology of TRALI and because its signs and symptoms frequently mimic those of other conditions, an updated scheme was proposed in 2019 by leading TRALI experts. The modification divides the condition into two types. Type I TRALI criteria are the following: acute hypoxemia with radiographically evident pulmonary edema and without signs of left atrial hypertension, onset of symptoms within 6 hours of transfusion, and the absence of risk factors for acute respiratory distress syndrome (ARDS) [21]. Type II TRALI refers to a scenario in which a patient who does possess a risk factor for ARDS develops worsening pulmonary status worsens in the setting of a transfusion [21]. As in the case in TACO, a two-hit phenomenon has been hypothesized to occur in the majority of cases of TRALI. The first hit is a priming event, in which endothelial activation causes increased neutrophil migration to the alveoli [22]. The inciting event is not always entirely clear, but examples include trauma, sepsis, surgery, and critical illness [23]. The second hit occurs with the transfusion. Antibodies and other mediators that have accumulated in stored products activate these neutrophils, triggering a proinflammatory cascade that ultimately results in pulmonary injury and edema [22]. Transfusion-related risk factors include administration of plasma and volumes of human leukocyte antigen class II antibody and anti-human neutrophil antigen [19]. Over the past two decades, screening for these antibodies as well as administration of male-predominant plasma has lowered the incidence of TRALI significantly, though it still remains the most common cause of transfusion-related deaths in the United States [23]. When TRALI is suspected, the clinician should pay particular attention to the patient's pulmonary status and be ready to support it. Supplemental oxygen or non-invasive support may be sufficient, but many patients will require endotracheal intubation. Clinicians should employ lung-protective strategies for intubated patients, as they would for patients with acute respiratory distress syndrome [24]. If TRALI is suspected, the transfusion service should be contacted without delay, in order to ensure that no subsequent units from the donor are transfused to other patients.

Conclusion

Advances in donor screening and transfusion protocols in recent decades have helped reduce the incidence of some but not all transfusion-related complications. Though many of the adverse events are relatively benign and do not harbor significant morbidity, they can result in patient anxiety and dissatisfaction, increased resource utilization, and even avoidance of transfusion. Accordingly, clinicians must exercise vigilance when electing to administer blood products to patients, be aware of the signs and symptoms of complications, and know how to treat them. Maintaining an open line of communication with blood bank personnel can aid clinicians in taking steps to minimize adverse events and in dealing with complications when they arise.

Key Points

- Transmission of hepatitis and HIV in the setting of blood product transfusions has decreased to near zero
- Signs and symptoms of various types of transfusion reactions can be similar and difficult to distinguish. The spectrum of severity ranges from asymptomatic to fatal
- Generally, immediate cessation of the transfusion, communication with blood bank personnel, and supportive care are the mainstays of initial management of suspected transfusion reaction. Further laboratory analysis can help narrow the diagnosis
- TRALI and TACO are both associated with high morbidity and mortality, particularly in patients with pre-existing co-morbidities who receive multiple units of blood products. Vigilance and stewardship are essential in the management of these patients

References

1. Zou S, Dorsey KA, Notari EP, et al. Prevalence, incidence, and residual risk of human immunodeficiency virus and hepatitis C virus infections among United States blood donors since the introduction of nucleic acid testing. Transfusion. 2010;50(7):1495–504.
2. Zou S, Stramer SL, Dodd RY. Donor testing and risk: current prevalence, incidence, and residual risk of transfusion-transmissible agents in US allogeneic donations. Transfus Med Rev. 2012;26(2):119–28.
3. Niederhauser C. Reducing the risk of hepatitis B virus transfusion-transmitted infection. J Blood Med. 2011;2:91–102.
4. Hong H, Xiao W, Lazarus HM, Good CE, Maitta RW, Jacobs MR. Detection of septic transfusion reactions to platelet transfusions by active and passive surveillance. Blood. 2016;127(4):496–502.
5. Delaney M, Wendel S, Bercovitz RS, Cid J, Cohn C, Dunbar NM, Apelseth TO, Stanworth SJ, Tinmouth A, Van de Watering L, Waters JH, Yazer M, Ziman A, Biomedical Excellence for Safer Transfusion (BEST) Collaborative. Transfusion reactions: prevention, diagnosis, and treatment. Lancet. 2016;388(10061):2825–36.
6. Panch SR, Montemayor-Garcia C, Klein HG. Hemolytic transfusion reactions. N Engl J Med. 2019;381(2):150–62.
7. Carson JL, Guyatt G, Heddle NM, Grossman BJ, Cohn CS, Fung MK, Gernsheimer T, Holcomb JB, Kaplan LJ, Katz LM, Peterson N, Ramsey G, Rao SV, Roback JD, Shander A, Tobian AA. Clinical practice guidelines from the AABB: red blood cell transfusion thresholds and storage. JAMA. 2016;316(19):2025–35.
8. Savage WJ. Transfusion reactions. Hematol Oncol Clin North Am. 2016;30(3):619–34.
9. Domen RE, Hoeltge GA. Allergic transfusion reactions: an evaluation of 273 consecutive reactions. Arch Pathol Lab Med. 2003;127(3):316–20.
10. Hirayama F. Current understanding of allergic transfusion reactions: incidence, pathogenesis, laboratory tests, prevention and treatment. Br J Haematol. 2013;160(4):434–44.
11. Tobian AA, Savage WJ, Tisch DJ, Thoman S, King KE, Ness PM. Prevention of allergic transfusion reactions to platelets

and red blood cells through plasma reduction. Transfusion. 2011;51(8):1676–83.
12. Goel R, Tobian AA, Shaz BH. Noninfectious transfusion-associated adverse events and their mitigation strategies. Blood. 2019;133(17):1831–9.
13. Cohen R, Escorcia A, Tasmin F, Lima A, Lin Y, Lieberman L, Pendergrast J, Callum J, Cserti-Gazdewich C. Feeling the burn: the significant burden of febrile nonhemolytic transfusion reactions. Transfusion. 2017;57(7):1674–83.
14. King K, Shirey R, Thoman S, Bensen-Kennedy D, Tanz W, Ness P. Universal leukoreduction decreases the incidence of febrile nonhemolytic transfusion reactions to RBCs. Transfusion. 2004;44(1):25–9.
15. Ning S, Solh Z, Donald MA, Morin P. Premedication for the prevention of nonhemolytic transfusion reactions: a systematic review and meta-analysis. Transfusion. 2019;59(12):3609–16.
16. Yazer MH, Podlosky L, Clarke G, Nahirniak SM. The effect of prestorage WBC reduction on the rates of febrile nonhemolytic transfusion reactions to platelet concentrates and RBC. Transfusion. 2004;44(1):10–5.
17. Centers for Disease Control. National healthcare safety network biovigilance component hemovigilance module surveillance protocol. https://www.cdc.gov/nhsn/pdfs/biovigilance/bv-hv-protocol-current.pdf. Accessed 29 Nov 2020.
18. Semple JW, Rebetz J, Kapur R. Transfusion-associated circulatory overload and transfusion-related acute lung injury. Blood. 2019;133(17):1840–53.
19. Popovsky MA. Transfusion-related acute lung injury: three decades of progress but miles to go before we sleep. Transfusion. 2015;55(5):930–4.
20. Kleinman S, Caulfield T, Chan P, Davenport R, McFarland J, McPhedran S, Meade M, Morrison D, Pinsent T, Robillard P, Slinger P. Toward an understanding of transfusion-related acute lung injury: a statement of a consensus panel. Transfusion. 2004;44(12):1774–89.
21. Vlaar APJ, Toy P, Fung M, Looney MR, Juffermans NP, Bux J, Bolton-Maggs P, Peters AL, Silliman CC, Kor DJ, Kleinman S. A consensus redefinition of transfusion-related acute lung injury. Transfusion. 2019;59(7):2465–76.
22. Vossoughi S, Gorlin J, Kessler DA, Hillyer CD, Van Buren NL, Jimenez A, Shaz BH. Ten years of TRALI mitigation: measuring our progress. Transfusion. 2019;59(8):2567–74.
23. Otrock ZK, Liu C, Grossman J. Transfusion-related acute lung injury risk mitigation: an update. Vox Sang. 2017;112(8):694–703.
24. Friedman T, Javidroozi M, Lobel G, Shander A. Complications of allogeneic blood product administration, with emphasis on transfusion-related acute lung injury and transfusion-associated circulatory overload. Adv Anesth. 2017;35(1):159–73.

The Effects of Hemoglobin-Based Oxygen Carriers (HBOC) on the Microcirculation

8

Anthony T. W. Cheung and Peter C. Y. Chen

Background

Hemorrhagic shock (caused by a severe loss in blood volume) can lead to irreversible organ damage or death in human patients if not appropriately managed and treated within a short (≤1 hour) time-frame. For severe blood loss, transfusion with allogeneic blood is a standard in-hospital management protocol and is considered the "gold standard" for treatment [1, 2]. However, when allogeneic blood is not available in out-of-hospital settings (as in rural or battlefield injuries), plasma expanders (including crystalloid and colloid solutions) are commonly used as an alternative [2–4]. Crystalloid solution includes saline and Ringer's solution while colloid solution includes hetastarch (Hespan®), bovine serum albumin (BSA) and artificial blood substitutes (e.g., hemoglobin-based oxygen carriers (HBOC)), in which the hemoglobin component acts as a colloid. Hemoglobin consists of four linked globulin chains and is the protein molecule in the red blood cells (RBC) which is responsible for transporting oxygen and carbon dioxide for gaseous exchange in the microcirculation of the tissues and organs. As a treatment for severe blood loss, hemoglobin cannot be transfused directly into the systemic blood circulation, as it will cleave into dimers and monomers, which are toxic to the kidney and will lead to irreversible damage. If one wants to study the effects of hemoglobin on hemorrhagic shock treatment, HBOC have to be used. HBOC represent a group of oxygen carrying hemoglobin molecules which are polymerized as tetramers and have no toxic effect on the transfusion recipients. A few generations of HBOC have been developed; although they were all hemoglobin-based, each one was designed and manufactured differently and might possess different adverse side-effect profiles. When one wants to study the effects of HBOC on the microcirculation, several HBOC from different generations should be investigated in order to generate a more extensive profile of results. In this chapter, we focused on the effects of four early generations of HBOC (Oxyglobin®, Hemoglobin Glutamer-200 (bovine)®, Biopure®, and Hemolink™) and a recent generation of non-hemoglobin based oxygen carrying artificial blood substitute (PEGylated RBC) on the microcirculation.

Hemorrhagic shock and transfusion treatment both can affect the overall functional profile of the cardiovascular system in general and the systemic circulation in particular. The effects on the systemic circulation can be divided into two categories; the macrocirculation and the microcirculation. As one can imagine, both circulations have different functional roles but work together seamlessly to maintain homeostasis of the body, and one circulation is just as important as the other. It is through the macrocirculation that oxygen supply and nutrients are transported to the tissues and organs, but it is important to realize that the oxygen and carbon dioxide gaseous exchange (in the lungs as well as in other tissues and organs) and all cellular functions actually take place at the microcirculation level, the *raison d'etre* of the circulation system (as appropriately described by Professor Marcos Intaglietta in his acceptance lecture for the Eugene Landis Award in the 1999 Annual Meeting of the Microcirculatory Society) [1]. The subject material in this chapter deals with the microcirculation in general, but with emphasis on the identification of landmark vasculopathy features which can serve as unique bio-markers to reflect the hematopathology and responses arising as the effects of hemorrhagic shock and HBOC transfusion as a treatment modality.

When we study the microcirculation *per se*, we will be dealing with an organized network of arterioles, venules and capillaries. A search in the literature revealed that one normally could not adequately study the blood flow characteristics and vasculopathy of all three types of micro-vessels in

A. T. W. Cheung (✉)
Department of Pathology and Laboratory Medicine, University of California, Davis School of Medicine, Sacramento, CA, USA

Institute for Biomedical Sciences, San Diego, CA, USA

P. C. Y. Chen
Institute for Biomedical Sciences, San Diego, CA, USA

Department of Bioengineering, University of California, San Diego, La Jolla, CA, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_8

the same microcirculation. Some microvascular beds might have a very high distribution of capillaries but with very few arterioles and venules (e.g., human nailfold, hamster skinfold), while other microvascular networks may be ideal locations to study arterioles and venules but not necessarily capillaries (e.g., human and canine bulbar conjunctiva). In addition, it is worthwhile to realize that the word "capillary" was used quite loosely referring to all micro-vessels in the literature and not just capillary alone. For example, the title of the seminal publication by Davis and Landau (*Clinical Capillary Microscopy*) consisted of still pictures showing vasculopathy features not necessarily in capillaries, but mostly in arterioles and venules [5]. In our laboratories at the University of California, Davis (UCD) and University of California, San Diego (UCSD), we have developed two different animal models for hemorrhagic shock and HBOC transfusion investigations; a hamster dorsal skinfold window chamber "exclusively" for capillary studies and a modified Wiggers hemorrhagic shock canine model "mostly" for arterioles and venules studies, but at times included capillaries to illustrate the entire vasculopathy picture.

The conjunctival microvasculature has often been selected as an easily-accessible location for microcirculation research. It is a well-organized microvascular bed with an orderly distributed network of arterioles, venules and capillaries. The interest in using the conjunctival microvasculature as a location to investigate disease severity and its correlation with vasculopathy was popularized in 1966, when Davis and Landau co-authored a seminal monograph, entitled "*Clinical Capillary Microscopy*", which featured a broad collection of 289 still pictures of the bulbar conjunctival, finger nailfold and sublingual microcirculations in human patients inflicted by various diseases; in reality showing a plethora of pathological features (vasculopathy) in the arterioles, venules and capillaries of patients for the first time in medical literature [5]. All the pictures possessed outstanding photographic quality; with good resolution in image display and well-defined landmark (unique) vasculopathy features for disease correlation. The only limitation in these pictures lay in the fact that they were one time still pictures and, as such, could not show any longitudinal and time-dependent changes in vessel morphometry, dynamics and disease progression. In other words, the still pictures were frozen in one specific moment in time and were inadequate for longitudinal (time-dependent follow-up) and real-time (on-screen changes during intervention treatment or disease progression) interpretations. The presence of unique landmark features in the vasculopathy caused by different diseases and their progressive development inspired us to design a video approach to record real-time events in the conjunctival microcirculation for subsequent longitudinal analysis. In order to eliminate the still picture limitations, we have designed and fabricated one small animal (rodent/hamster) skinfold-dedicated and two large-animal (human and dog) bulbar conjunctiva-dedicated computer-assisted intravital microscopes (CAIM) to observe and continuously record dynamic and morphometric events and longitudinal (time-dependent) activities in the microcirculation. Other than being selected solely as an easily-accessible real-time study location (one of only three available in human studies), the conjunctival microvasculature was actually chosen for relevant anatomical and physiological reasons. The bulbar conjunctiva has always been recognized as an integral component of the ocular surface of the eye, and the bulbar conjunctival microcirculation arises directly from the carotid artery -- in fact it is often viewed as a terminal microvascular bed of the intracranial carotid artery [6, 7]. This anatomical link suggests strongly that a correlation exists between the conjunctival and intracranial vasculopathy. This link is viewed as a path (a "worm hole" so as to speak), which can lead directly to an understanding of activities in the brain itself as well as the intracranial arteries in particular; literally using the eye as an outside window to look inside the cranium. This relationship and the importance of the conjunctival microcirculation have been independently confirmed by an unrelated study at the UCD Medical Center and Oakland Children's Hospital as part of the 1998 Cooperative Study of Sickle Cell Disease (CSSCD) -- a double-blinded multiple-center study for stroke vulnerability and prediction in the US, as described in a related published report; "*Correlation of intracranial vessel velocity (measured by transcranial Doppler ultrasonography) with abnormal conjunctival vessel velocity (measured by computer-assisted intravital microscopy) in sickle cell disease*" [8]. This National Institutes of Health (NIH) funded CSSCD study established the physiological relevance and importance of the "extracranial" conjunctival microcirculation in vasculopathy studies to predict "intracranial" strokes in juvenile patients, and validated the abnormal blood flow velocity in the conjunctival microcirculation as an independent bio-marker to predict vulnerability of juveniles with sickle cell disease to obstructive strokes in the intracranial arteries located at the Circle of Willis [8]. The fact that the recently-developed CAIM technology successfully correlated with the well-established and clinically-accepted Doppler ultrasonography technology further validated the clinical acceptance of this novel technology and significantly enhanced the reliability and creditability of this real-time study on the microcirculation.

In addition, most conjunctival arterioles and venules have different key landmark features and contours (e.g., vessel shape like tortuosity, unique capillary or arteriolar blood-flow pattern like box-car trickled flow, sludging and bulk flow, damaged vessel, wide vessel diameter, branching, vessel distribution, hemosiderin deposits, etc.) which can be identified and relocated for follow-up studies (see some of the unique features in Fig. 8.1c, d). In other words, one can

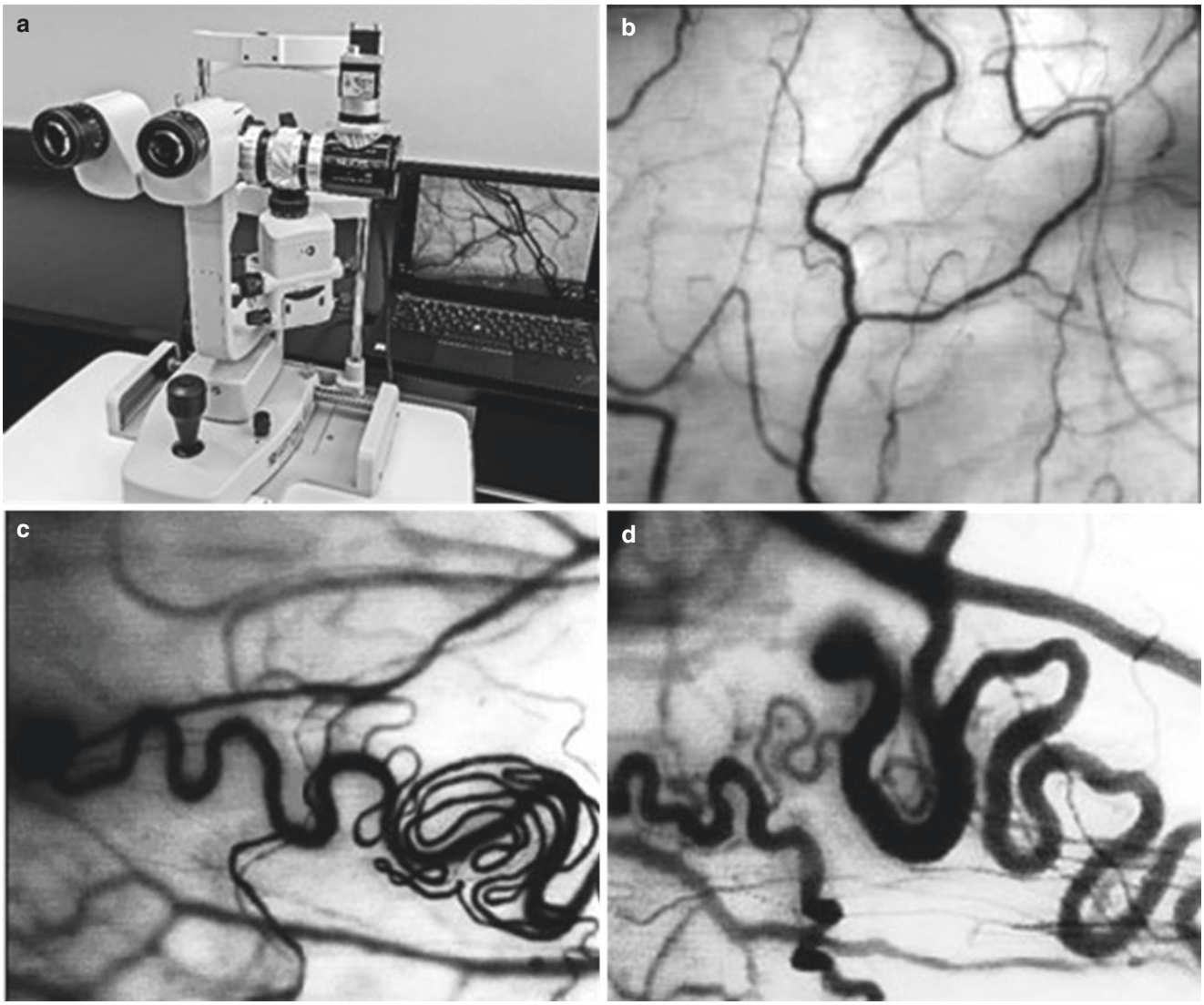


Fig. 8.1 (**a**) A slit-lamp based CAIM system which was fabricated and adapted for use at UCD Medical Center and Oakland Children's Hospital for the NIH-CSSCD multiple-center clinical trial and UCD multiple-disease study. (**b**) A typical (text-book) frame-captured image of the conjunctival microcirculation in a healthy adult volunteer with no history of vascular disease. Note the even and orderly distribution of normal-sized arterioles, venules and capillaries in a well-organized network. (**c**) A view of the conjunctival microcirculation from a patient exhibiting severe vasculopathy. Note the chaotic organization of arterioles and venules, the uneven presence of capillaries, and the abnormal but unique shape and size of many vessels. The unique shape of any vessel could be used as key reference to identify and relocate the same vessel for follow-up studies – using each vessel as its own reference control. Note also the unique tortuosity of a large vessel located in the middle of the field. This abnormal sinusoidal configuration was indicative of and diagnostic for hypertension in the patient. The pressure build-up in this hypertensive vessel was very high and caused a recent damage at its far end on the left side of the field, with blood leaking out from the damaged area. (**d**) A view of the conjunctival microcirculation from another patient. All vessels (venules) exhibited abnormally wide diameters. In addition, one could see the recovered end of a damaged vessel in the middle of the field. The distribution of capillaries was very uneven and a significant absence of capillaries occurred at the upper right corner of the figure. (For Fig. 8.1b–d, the optical magnification was 4.5×)

investigate changes in most micro-vessels in the conjunctival microvasculature in disease/vasculopathy studies longitudinally, using each vessel as its own baseline reference (see Fig. 8.3c, d). Meaningful correlations have also been established between the unique vasculopathies with disease severity and *clinical sequelae* of the patients in a multiple-disease IRB-approved study in UCD Medical Center on diabetic (type-1 and type-2), hypertensive, sickle cell and Alzheimer's disease patients, simultaneous pancreas-kidney transplanted diabetic patients, and myopic contact-lens users [8–19]. Despite our capability and success in using CAIM to study the conjunctival microvasculature in patients, it is not ethical or legally-permitted for any scientist or clinician to conduct experimental or invasive studies (e.g., intentional induction

of severe blood loss and experimental transfusion) in human volunteers solely for the purpose of collecting experimental data. In view of these concerns, intervention studies designed to investigate the real-time effects of HBOC on the microcirculation can best be performed in a hamster dorsal skinfold window chamber and canine hemorrhagic shock model in conjunction with the microscopic and video capability of CAIM.

Effects on the Microcirculation: A Hamster Skinfold Study

The hamster dorsal skinfold window chamber has been developed and utilized successfully in UCSD by Intaglietta, Tsai, Chen, Cheung, Winslow et al., (USA) and Messmer et al., (FRG) to study hemorrhagic shock, hemodilution, plasma expansion, capillary perfusion, capillary flow impairment, oxygenation, functional capillary density (FCD), HBOC design, and effectiveness of HBOC as oxygen carrying plasma expanders [1, 4, 20–30].

A modified version of the dorsal window chamber was implanted in a Syrian golden hamster under anesthesia (sodium pentobarbital/Nembutal) as described in a previous report [4]. Catheters were inserted into the carotid artery and jugular vein for transfusion and blood withdrawal for blood chemistry measurements. All hamsters were allowed to recover for 48 hours before use. During experimentation, each fully recovered and awake hamster was placed in a customized Plexiglas holder with the window positioned directly under the nose-piece of the CAIM system for observation and video-recording with a COHU CCD video camera as shown in Fig. 8.2a–d and described previously [4].

In this capillary oriented transfusion study, we utilized the latest generation of oxygen-carrying artificial blood substitute, PEGylated RBC, which was significantly different from the earlier generations of HBOC. Twenty-five hamsters were transfused with various concentrations of PEGylated RBC (Cerus Corporation, Concord, CA, USA), and four hamsters were transfused with allogeneic (donor) blood (i.e., hamster RBC without PEGylation) as control. Prior to the top-loading transfusion of PEGylated RBC, the blood hematocrit, total

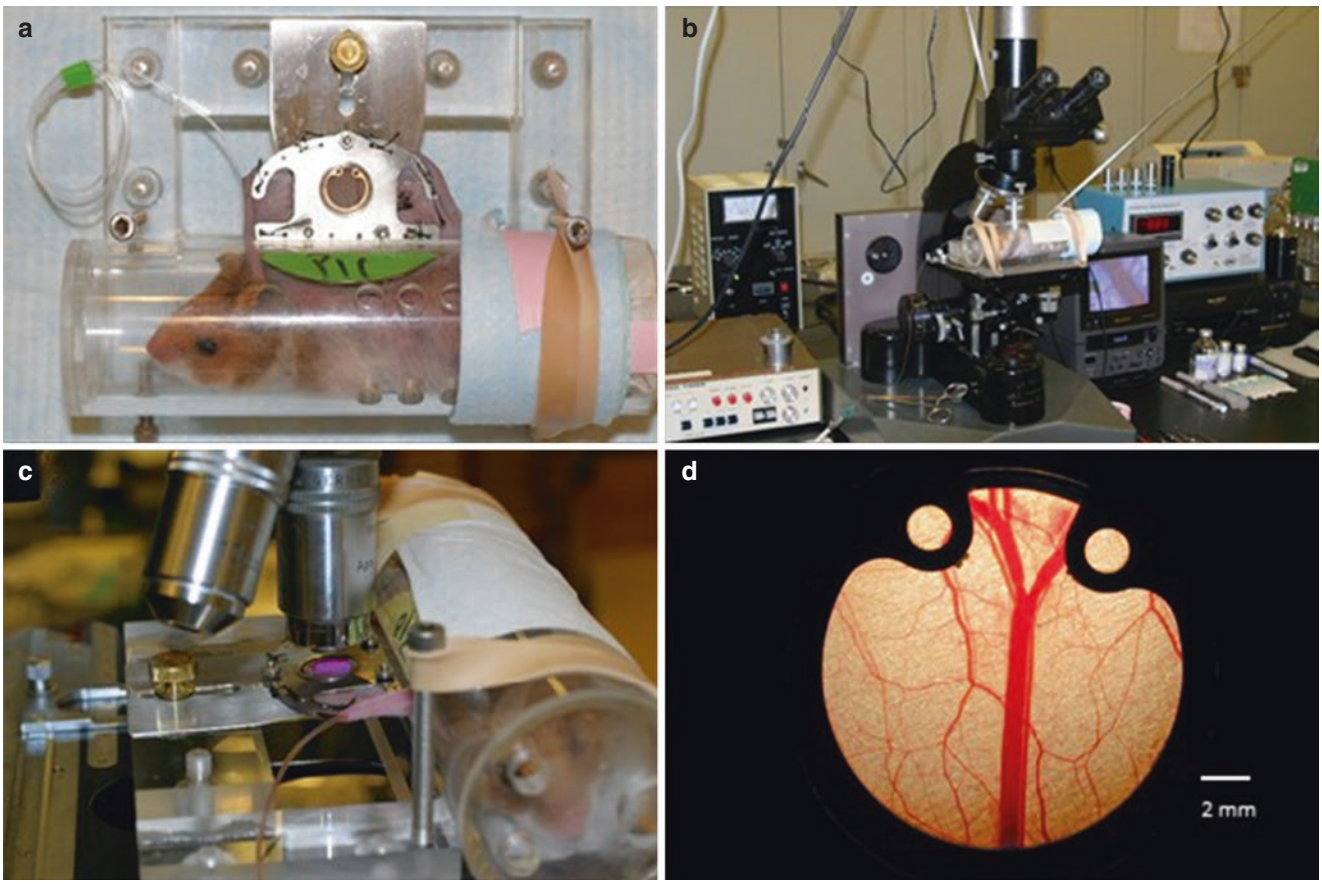


Fig. 8.2 (**a**) A hamster with the dorsal skinfold window chamber installed and resting in a custom Plexiglass holder. (**b**) Holder with hamster mounted onto the Leitz intravital microscope specimen stage. (**c**) Observation of the window chamber under the microscope nose-piece. (**d**) Representative view of the hamster skinfold microcirculation as seen through the window. Note that a large majority of the vessels were capillaries. There was only one venule in view (the central vessel with a wide diameter) with a pairing arteriole by its side

blood hemoglobin and oxygenation (pO_2) were measured as baseline reference. After the vital signs and measurements had stabilized, a solution (equal to 10% of the calculated total blood volume of the recipient hamster) of PEGylated RBC in concentrations of 0.05 mM-22-mM with a 5 kDa conjugate and concentrations of 0.05–5 mM with a 20 kDa conjugate was transfused via the carotid artery catheter into the hamster. The response of the microcirculation to the top-loading transfusion was viewed through the window and recorded for up to an hour. The blood hematocrit and total hemoglobin were measured again (using withdrawn venous blood samples) at the end of the video-recording while pO_2 was monitored continuously. Selected video sequences were measured for vessel diameter and blood flow velocity to compare with pre-transfusion baseline values so as to determine the effects of PEGylated RBC on the microcirculation. The results showed a minor non-significant increase in the total blood volume and hematocrit and no noticeable change in total blood hemoglobin after the transfusion. The pO_2 remained essentially unchanged throughout the study. The result was surprising since PEGylated RBC was constructed as an oxygen carrier. This observation led to the suspicion that the oxygenation measurements did not adequately reflect the tissue pO_2 level in a short time-frame when oxygen unloading was involved with an artificial blood substitute on the tissue level [2, 3]. As for the real-time effects on the microcirculation, noticeable but non-significant changes in the diameter of the capillaries and blood flow velocity occurred. In conclusion, a 10% top-loading transfusion resulted in only marginal non-significant effects on the skinfold microcirculation and blood chemistry. If a volume-to-volume transfusion was performed, hemorrhagic shock would have been established and the transfusion results might be different. It was interesting to note that this top-loading transfusion study did confirm that PEGylated RBC were non-toxic when used in a transfusion, which was well-tolerated by the hamsters. In view of the results, a volume-to-volume exchange transfusion (large blood volume withdrawal to establish hemorrhagic shock and equal volume transfusion as treatment) on a large animal, using the canine hemorrhagic shock model, would be conducted as a follow-up study to further understand the transfusion effects on the microcirculation.

Intaglietta, Tsai, Winslow, Messmer, et al., have extensively utilized the hamster dorsal skinfold window chamber to study top-loading and exchange HBOC transfusions and have contributed immensely to our knowledge and understanding on the effects of HBOC on capillary function [24–28, 31, 32]. The results on their top-loading transfusion studies were akin to the results in our hamster investigation. However, their greatest contributions lay in their long-term collaborative studies on the post-transfusion microcirculation, which led to an in-depth understanding of hemodilution, plasma expansion, tissue oxygenation, and the realization on how the physical properties of blood, plasma expanders as well as HBOC could affect microvascular function and capillary integrity [1, 20–30, 33]. When considered globally, their collective contribution was instrumental in identifying the basic flaw in the design of the earlier generations of HBOC in that their effects on the microcirculation have not been anticipated nor appreciated, and that these earlier HBOC have not been "engineered" so that their physical properties would be congruent with those of the microcirculation [1, 25, 28]. In addition, it has been shown that the presence of these HBOC in the plasma spaces resulted in the paradoxical effect of producing vasoconstriction and reducing tissue oxygenation, an outcome opposite to what was needed for a patient in hemorrhagic shock [25, 29, 30]. The design flaws they identified and the results from their hemodilution, plasma expansion and oxygenation studies could now provide a valuable knowledge vault, consisting of an in-depth understanding on what to avoid and how to "engineer" a new generation of functional HBOC which will not inadvertently lead to paradoxical responses or fatal outcome [24, 25, 28].

Effects on the Microcirculation: A Canine Bulbar Conjunctiva Study

In most teaching curriculum on physiology for first year medical students, a core of interrelated lectures and canine-based laboratory sessions are dedicated to allow medical students to have hands-on experience in working with OR monitoring instrumentation and life-support devices, and to generate an in-depth understanding of and appreciation for physiological concepts, as well as to interpret canine organ functions, cardiovascular-pulmonary activities and vital measurements for human extrapolation and interpretation. In UCD School of Medicine, we have extended our curriculum to include a special hemorrhagic shock session, which is based on the adaptation of the modified Wiggers canine model in a fully-equipped OR setting [2, 3, 34]. Cardiovascular, pulmonary, oxygenation and blood chemistry changes during hemorrhagic shock and shed blood transfusion as a treatment modality are the main themes of this session. Microcirculation studies are not included as a part of this hemorrhagic shock session.

However, for research purposes and not for teaching, our laboratories have further extended this curriculum concept and adapted the modified Wiggers canine model in conjunction with the CAIM technology in an in-hospital OR setting to study hemorrhagic shock (induced by ≥50% blood loss) and the real-time effects of HBOC transfusion-treatment on the microcirculation [2, 3].

A total of 10 dogs were used in this real-time OR-based microcirculation study; six for Oxyglobin® transfusion and

four for shed blood (autologous) transfusion as control. Anesthesia was induced with IV propofol (2–4 mg/kg) and diazepam (0.5 mg/kg) followed by orotracheal intubation. A stable anesthetic plane was maintained using isoflurane and fentanyl to minimize confounding hemodynamic effects. Each anesthetized dog was splenectomized and allowed to stabilize for an hour. The amount of sequested blood in the spleen was collected, measured and included in the calculation of total blood volume. At the end of an one-hour stabilization period, pre-hemorrhagic (baseline) measurements on systemic-bodily functions, oxygenation and other vital activities were made (pre-hemorrhagic phase). In addition, video-recordings were made on the conjunctival microcirculation via CAIM simultaneously to serve as a baseline for later analysis, comparison, and correlation. Pre-hemorrhagic baseline systemic-bodily functions, oxygenation, blood hematocrit, total and blood hemoglobin, and microcirculation measurements (vessel diameter, vessel distribution, and blood flow velocity) were comparable in all 10 dogs. The diameter of all vessels averaged from all 10 dogs (arterioles 27 ± 9 μm; venules 43 ± 12 μm; capillaries ≤10 μm; n = 10 from each dog and then averaged between 10 dogs) were quantified by image analysis. Immediately after the baseline measurements and video-recordings were made, a large amount of the calculated total blood volume (based on body weight and including the amount of sequested blood in the spleen) was withdrawn from the lateral saphenous and femoral veins at a rate of 32–36 mL/kg/hr. until a targeted mean arterial pressure (MAP) of 45–50 mm Hg was achieved with the volume of withdrawn blood amounting to ~50% of the total blood volume (a clinical criterion targeted to establish acute but non-fatal hemorrhagic shock). This induced blood loss would take about 45 minutes to reach the targeted MAP. The shed blood of the four dogs was collected to be used in autologous transfusion as control. Within a one-hour acclimatization period (post-hemorrhagic phase) from the end of blood withdrawal, similar measurements and video-recording akin to the pre-hemorrhagic phase were made (post-hemorrhagic measurements). Special care was taken to assure the identification of the same vessels recorded in the pre-hemorrhagic phase for post-hemorrhagic video-recording. At the end of the post-hemorrhagic phase, IV transfusion using Oxyglobin® (at a rate of 10 mL/kg/hr. for 1 hour) for the six experimental dogs and shed blood (at a rate of 30 mL/kg/hr for 1 hour) for the four control dogs were made. Post-transfusion measurements and video-recordings were carried out at the end of the one-hour transfusion process (post-transfusion phase). The effects of hemorrhagic shock and subsequent transfusions (shed blood transfusion as control and Oxyglobin® transfusion as a treatment modality) on the microcirculation are summarized as follows.

1. Immediately after completion of ~50% hemorrhaging (blood withdrawal), all 10 dogs remained stable under anesthesia with no significant changes in body temperature, heart rhythm, respiratory rate, cardiopulmonary characteristics, and ventilatory parameters. MAP was significantly reduced to 45–50 mm Hg as targeted. In addition, all 10 dogs showed similar significant ($P \leq 0.01$) changes in systemic functions and oxygenation characteristics, including decreases in blood hematocrit, plasma and total hemoglobin, SpO_2 and increases in heart rate, respiratory rate and lactic acidosis. Heart rate and respiratory rate started to increase at the beginning of the hemorrhaging and substantially increased during the one-hour acclimatization process, resulting in a significant ($P \leq 0.01$) increase of ~20–25% at the end of 1 hour. Cardiac rhythm (ECG) remained normal except for noticeable tachycardia. Significant ($P \leq 0.01$) post-hemorrhagic changes also occurred simultaneously in the microcirculation of all dogs, including a ~20% decrease in venular diameter but with no significant change in arteriolar diameter. The presence of capillaries diminished noticeably during the entire process of blood withdrawal. In addition, a significant ($P \leq 0.01$) ~30% increase in blood flow velocity occurred. These significant changes ($P \leq 0.01$) in the microcirculation were compensatory changes which were indicative of sympathetic effects arising from severe blood loss.
2. Following **shed blood transfusion**, which was initiated 1 hour after the completion of severe (~50%) blood loss to ensure the establishment of hemorrhagic shock, all four control dogs restored systemic functions and oxygenation to pre-hemorrhagic (baseline) values – the significant decreases in blood hematocrit, total and plasma hemoglobin and SpO_2 values reversed to normalcy. The post-hemorrhagic increase in heart rate, respiratory rate and lactic acidosis also returned to baseline values. In addition, the significant post-hemorrhagic ~20% decrease in venular diameter and ~30% increase in blood flow velocity also reversed to baseline values. The capillaries, which had disappeared after severe blood loss, started to reappear in the beginning of the transfusion and reached ≥90% normalcy at the end of the transfusion. The results further confirm the understanding that allogeneic (autologous if available) blood transfusion is recognized as the "gold standard" for a successful hemorrhagic shock treatment.
3. **Oxyglobin® transfusion** induced no apparent change in the blood hematocrit but caused a noticeable but non-significant increase in the blood plasma and total hemoglobin values. Heart rate, respiratory rate and lactic acidosis reversed to 85–90% of pre-hemorrhagic baseline values. Surprisingly, SpO_2 level did not change significantly and remained low although Oxyglobin® was an

oxygen carrier by design. As for the post-transfusion effects on the microcirculation itself, the changes in post-hemorrhagic venular diameter and blood flow velocity significantly ($P \leq 0.01$) reversed to near-baseline values. The capillaries which had disappeared after severe blood loss reappeared as in shed blood transfusion. The effects on the microcirculation did not appear to be caused by any specific chemical benefit provided by the artificial blood substitute, but rather occurring as a result of the infusion of a plasma expander (i.e., significant increase in viscosity and fluid volume) into the systemic circulation in the transfusion process. When the Oxyglobin® transfusion was conducted by the end of the one-hour post-hemorrhagic acclimatization period (the golden hour), the reversal of vessel diameter and blood flow velocity persisted after a follow-up observation period of at least 2 hours.

4. If the usually successful shed (autologous) blood transfusion was delayed and conducted beyond the one-hour time-frame after the establishment of hemorrhagic shock (e.g., similar to hours of delay in ambulance transport or battle-field injury), marginal reversal of changes on heart rate, respiratory rate, oxygenation, acidosis, MAP, and micro-circulation would take place for a short period of time. However, the reversal changes leveled off in ~20–25 minutes and all adverse effects arising from hemorrhagic shock progressively reappeared and persisted -- eventually leading to the ultimate demise of the dog from microcirculation failure and hemorrhagic shock complications. Apparently, this one-hour time-frame was an important determinant for a successful or non-successful transfusion outcome. The importance and significance of this "golden hour" could not be overlooked. In addition, the real-time changes in the microcirculation during hemorrhagic shock and the post-transfusion reversals could serve as independent on-the-spot bio-markers to predict or confirm a successful or non-successful transfusion outcome.
5. Additional HBOC transfusion studies using **Hemoglobin Glutamer-200 (bovine)®, Biopure® and Hemolink™** were conducted in like manner to show the real-time effects of three additional HBOC. Comparable results akin to the effects generated by Oxyglobulin® were achieved, illustrating clearly that all four HBOC have identical effects on the microcirculation, as well as the consistency in our experimental results and reliability of our protocol using CAIM.

Discussion

During hemorrhagic shock, blood flow and intraluminal blood pressure in the microcirculation of tissues and organs are drastically reduced, creating a progressive oxygen-debt that will eventually become irreversible [1, 35]. Maintaining the integrity and preservation of the microcirculation in tissues and organs is of utmost importance to provide a shock patient a chance to survive. During hemorrhagic shock arising from a severe blood loss, the patient will progressively become very hypoxic. In the absence of adequate oxygen and carbon dioxide gaseous exchange at the microcirculation level where all cellular functions and activities take place at the same time, local respiration and metabolism will become anaerobic, and the microcirculation will start to lose its integrity and begins to fail [35]. If no appropriate treatment (allogeneic blood or plasma expander transfusion) is given immediately or soon (within ~one-hour time-frame), the microcirculation will fail completely and irreversibly, leading to the demise of the shock patient [35]. This pathologic course-of-event serves to explain the "golden hour" criterion for a successful survival outcome.

The basic principle and understanding of this "golden hour" to ensure a successful transfusion and ultimate survival in the canine model have been extrapolated to human interpretation successfully. However, misuse of this "golden hour" criterion did contribute to a few unfortunate and paradoxical episodes with unexpected results. During the long trips to the hospital from far-away rural areas, vasopressin (aka arginine vasopressin, a potent vasoconstriction agent) has often been administered as a pre-hospital (*en route*) treatment medication to re-distribute blood flow, prevent tissue hypoxia and improve cardiac output in hemorrhagic shock patients; with the expectation to prevent complications arising from the loss of microvascular integrity and ultimate failure of the microcirculation beyond the "golden hour" time-frame. However, it has been reported that, in some cases, vasopressin treatment might have led to unexpected adverse effects as evidenced by occasional post-vasopressin necrotic skin lesions and gastrointestinal ulcerations [36, 37]. Using the canine hemorrhagic shock model, CAIM has revealed that this type of ischemic lesions resulted from a severe reduction of blood flow arising from random but extensive post-vasopressin vasoconstriction and a significant loss of capillary function in the microcirculation [31]. Post-vasopressin video sequences focused on the same pre-vasopressin micro-vessels in the same locations (see Fig. 8.3c, d) revealed clearly that instead of redistributing blood flow to improve tissue oxygenation and avoid tissue hypoxia, vasopressin treatment has paradoxically caused extensive multi-focal vasoconstrictions in the microcirculation, resulting in a drastic cessation of blood flow and leading to severe tissue ischemia and pathogenesis [31]. In this study, the significantly reduced blood flow in the arterioles, venules and capillaries in the conjunctival microcirculation, which displayed a consistent vessel distribution and blood flow pattern before the vasopressin treatment as illustrated in Fig. 8.3c, constricted severely and some vessels even disap-

peared from view because of the absence of blood flow -- creating a "blanched" anemic appearance on the surface of the bulbar conjunctiva as illustrated in Fig. 8.3d and described in a previous report [31]. In most cases, the exact real-time focal lesions (i.e., point of constriction, shrinkage of vessel diameter, capillary disappearance, etc.) could be pin-pointed in the video sequences and frame-captured pictures as shown in Fig. 8.3d. This vasopressin study was actually a direct replica of our study on the real-time effects of HBOC on the microcirculation. The only difference was instead of using HBOC, we used this established CAIM technology translationally to study a pre-hospital (*en route*) medication to demonstrate its inadvertent effects. Again, it is easy to realize that this type of longitudinal studies could not be conducted without the unique landmark features of the arterioles, venules and capillaries (for same location identification and relocation) and the CAIM technology.

With the availability of the conjunctival microvasculature as a research platform and the incorporation of the CAIM technology as illustrated above, our laboratories have in real-

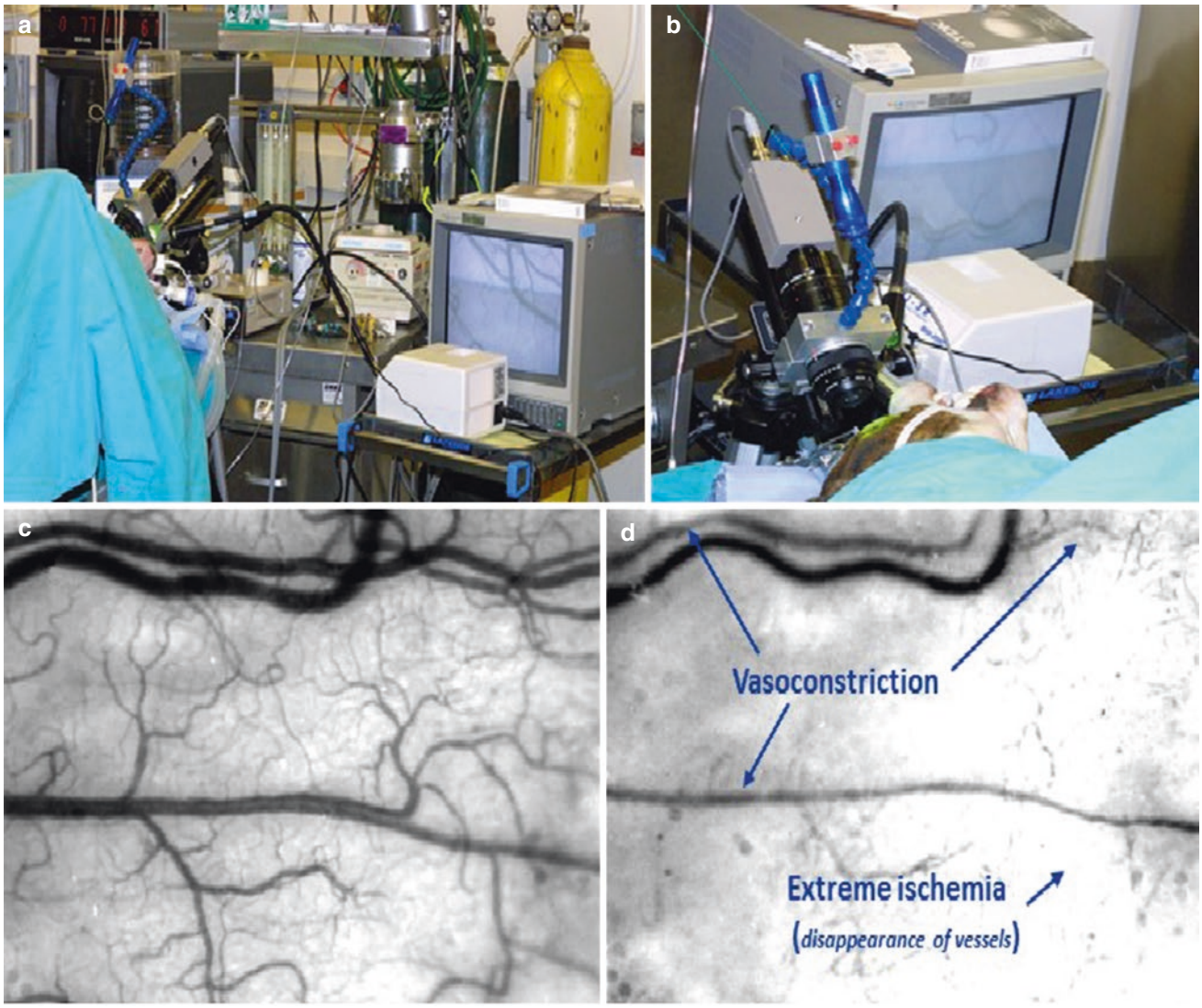


Fig. 8.3 (**a**) A section of the fully equipped OR-based bio-station. The COHU CCD video camera was positioned near the bulbar conjunctiva of the eye and aligned with the conjunctival microcirculation for precise focusing. (**b**) A close-up view of the video camera focusing on the bulbar conjunctiva with an image of the conjunctival vessels being displayed on the monitor screen. (**c**) A view of the normal conjunctival microcirculation of the dog before vasopressin treatment. A well-organized network of arterioles, venules and capillaries could be seen in sharp focus. (**d**) A view of the same conjunctival vessels in Fig. 8.3c after the vasopressin treatment. Extensive constrictions in most of the vessels have taken place, with many small branches of the arterioles and venules disappearing from view because of the cessation of blood flow. In addition, basically all the capillaries have disappeared because of the absence of active blood flow. The usefulness of identifying and relocating the pre-vasopressin (baseline) vessels for post-vasopressin follow-up comparison (literally using the vessel itself as its own reference control) significantly enhanced the accuracy and reliability of data acquisition. (For Fig. 8.3c, d, the optical magnification was 4.5×)

ity developed a fully OR instrumentation-based objective bio-station (see Fig. 8.3a, b) to study future artificial blood substitutes and to test the efficacy of medications and effectiveness of treatment protocols in a real-time setting, as in Flocor® (human study) and vasopressin (canine study) treatment [18, 31]. In fact, encouraged by the successful outcome in the study on the real-time effects of Oxyglobin®, our laboratories have extended further studies to include three additional HBOC (Hemoglobin Glutamer-200 (bovine)®, Biopure® and Hemolink™) in like manner. It was interesting to confirm that all three additional HBOC generated comparable effects on the microcirculation akin to the same outcome as Oxyglobin®, thus confirming the reliability of our CAIM studies. We have also made good use of the canine hemorrhagic shock model to evaluate the effects of contaminants (e.g., lead, lactate) on the functionality of HBOC [32, 38–40] and medication efficacy in microcirculation-involved applications [18, 31, 41].

The successful establishment and utilization of the canine model led to the design of other animal models for hemorrhagic shock and microcirculation studies. Recently, our laboratories have successfully established a rabbit hemorrhagic shock model to further study the effects of the most recent generation of PEGylated plasma expander (EAF-Hexa-PEG albumin (EAF-PEGylated BSA)) on the microcirculation, and to compare its effectiveness with PEGylated RBC, and crystalloid and colloid solutions in transfusion treatment [42].

Historically, the development of artificial blood substitutes could be traced back to the use of bovine serum albumin as a non-oxygen carrying plasma expander to treat severe blood loss in the battlefield in WWII. The necessity to develop a better plasma expander for rural injury and battlefield use, and hopefully also incorporating favorable oxygen-carrying and unloading characteristics in its development, was deeply ingrained in the mind of all on-the-field medical personnel and emergency room clinicians. This necessity and subsequent goals led to the design and manufacture of the first few generations of HBOC. These products were designed to serve as non-toxic bovine hemoglobin-based oxygen carriers, as in Oxyglobin®, Hemoglobin Glutamer-200 (bovine)®, Biopure®, and Hemolink™, to be used in emergency transfusion as a possible treatment modality for hemorrhagic shock in the 1970s through 1990s. Hemoglobin (bovine) was used as the backbone of these early blood substitutes because of their oxygen-carrying property, immunological profile and availability. In addition to research focusing on toxicity, additional emphasis was placed mostly on their chemistry, biochemistry and oxygenation characteristics; but the real-time effects of HBOC on the recipients' microvasculature and the resulting microvascular changes or possible failure have been ignored or rarely investigated. The outcome from using these blood substitutes in transfusion treatment has been disappointing. In fact, HBOC were proven to be detrimental at times and had inadvertently led to some fatalities when used in emergencies to manage severe blood loss [1]. The dismal outcomes resulted from a flaw in their design in not appreciating the importance of the microcirculation, which was the crucial site where cellular functions and activities would take place, and was also the site where hypoxia, anaerobic respiration and metabolism, and the ultimate failure of the tissues and organs would occur. In the past two decades, a more advanced and better-designed generation of artificial blood substitute was produced using a new conjugation technology. Instead of using the polymerization process to construct HBOC which were basically polymerized hemoglobin tetramers, the latest generation of blood substitute (PEGylated RBC and PEGylated BSA) was manufactured by conjugating polyethylene glycol (PEG) with red blood cells (RBC) or bovine serum albumin (BSA), resulting in new products which possessed good plasma expansion characteristics (including a high viscosity), fluid and pressure retention properties and a complete elimination of immunological complications irrespective of the difference in blood types in human RBC. Basically, most if not all PEGylated RBC employed human RBC as a building block because of their unmatched availability when blood bank-stored RBC supplies approached the end of their legally-permitted shelf-life. Again, the lack of an in-depth understanding on the effects of the PEGylated products on the microcirculation of the transfusion recipients still persisted. With our experience in utilizing the modified Wiggers canine hemorrhagic shock model and the incorporation of CAIM, real-time effects of the latest generation of PEGylated RBC and PEGylated BSA on the microcirculation can easily be investigated in our laboratories. In addition, the foundation to study PEGylated RBC and PEGylated BSA has been laid down already in previous studies in our laboratories (4,42). In these studies, the identification of unique on-the-spot bio-markers in the microcirculation, which reflected the results and effectiveness of HBOC and PEGylated product transfusions, has been established to serve as key landmark references for future PEGylated product efficacy evaluation and effective outcome determination [2–4, 32, 41, 42].

For any translational research-oriented scientist and clinician, to be able to effectively utilize results and technologies arising from animal research for human extrapolation and, better still, to serve as a stepping stone for direct human application, represents a highly targeted goal for their research endeavors -- as their search for the "holy grail" so as to speak. In addition to focusing solely on animal studies, our laboratories have enhanced and translationally adapted CAIM to be used non-invasively in a UCD IRB-approved multiple-disease clinical study on the conjunctival microcirculation in patients -- to identify relevant disease-related landmark vasculopathy features to supplement and correlate

with clinical evaluation, prediction, diagnosis and prognosis [7–18]. This multiple-disease IRB-approved clinical study was initiated in UCD Medical Center after the proven success in utilizing CAIM to record (videotape) conjunctival activities and vasculopathy for hemorrhagic shock correlation in the canine studies [2, 3]. These multiple-disease clinical studies have ultimately established various landmark criteria to serve as bio-markers for disease prediction and early diagnosis [8–18]. In a study on hypertension, the appearance of a unique vasculopathy feature -- vessel tortuosity -- has been accepted as a unique (landmark) bio-marker to diagnose hypertension in a patient (see Fig. 8.1c, d). In fact, the late Professor Benjamin Zweifach at UCSD identified this unique tortuosity feature to be the result of a sustained tissue remodeling process arising from a prolonged insult by high intraluminal blood pressure on the endothelial wall of the vessels, and appropriately named these "tortuous" vessels as "hypertensive" vessels. In addition, we have successfully studied the conjunctival vasculopathies in patients suffering from various diseases (type-1 diabetes, type-2 diabetes, hypertension, sickle cell and Alzheimer's disease, and ocular complications in contact lens users, just to name a few) [8–17]. As a case in point as described earlier, CAIM has been successfully utilized in the prediction of an intracranial stroke to occur in juvenile sickle cell disease patients, using the significant change in blood flow velocity in the extracranial conjunctival vessels as a bio-marker to predict a pending stroke in the intracranial vessels at the Circle of Willis (a clinically-accepted diagnostic interpretation by radiologists) in the CSSCD multiple-center stroke prediction study [8]. CAIM has also been utilized in a successful double-blinded clinical trial to confirm the efficacy of Poloxamer 188 (Flocor®) as a medication to treat vaso-occlusion during a sickle cell disease painful crisis [18]. Recently, we have used CAIM to confirm the association of plasma homocysteine level with vasculopathy in the conjunctival vessels of juvenile and adult sickle cell disease patients [19].

In conclusion, the incorporation of CAIM as an objective and quantitative tool in a clinical facility establishes the availability of a robust objective device in microcirculation research for clinical studies in patients. The identification of independent unique landmark bio-markers from CAIM-based conjunctival microcirculation studies can contribute significantly to a quick patient triage, disease prediction, early detection, diagnosis, severity determination, and a reliable outcome prognosis. ***Image how a quick triage assignment relying on a conjunctival bio-marker based diagnosis of a pending intracranial stroke, can lead to an immediate referral of a 6-years old sickle cell disease patient to life-saving emergency surgery, stem-cell transplant or gene therapy; events which will provide the most rewarding, satisfying and heart-warming feeling and a sense of achievement for any research scientist -- a life saved and a job well done.*** One can expect the same success and impact of future CAIM-based microcirculation studies on hemorrhagic shock, medication efficacy studies and new generations of artificial blood substitute effectiveness evaluations.

Key Points

- The bulbar conjunctival microvasculature is a direct continuation of the intracranial carotid artery.
- Conjunctival arterioles and venules have unique shapes and forms (e.g., tortuosity, branching); enabling them to be relocated for longitudinal studies – each vessel can serve as its own reference control.
- Disease severity and progression can be correlated with conjunctival vasculopathy for prognosis and timely triage.
- The most effective treatment modality for hemorrhagic shock is allogeneic blood transfusion.
- After a severe blood loss (40–50%), allogeneic blood transfusion needs to be conducted within an hour from the time of establishment of hemorrhagic shock to be effective (the golden hour).

Acknowledgments We would like to extend our heart-felt appreciation for the clinicians and nurses in the UCD Medical Center and Oakland Children's Hospital, the patients and their families -- without their assistance and support, the CSSCD and the multiple-disease clinical studies could not have come to fruition. In addition, the contribution from the pathology fellows, research fellows, graduate students, and machine shop specialists at UCD and UCSD is hereby gratefully acknowledged. The assistance from Dr. Adonis Stassinopoulos and Cerus Corporation (Concord, CA, USA) is very much appreciated. We thank the UCD Pathology Edmondson Fellowship, Howard Hughes Medical Institute SHARP Fellowship, two University of California Professional Development Awards (to ATW Cheung), American Heart Association Grant AHA0050789Y, Flocor® Phase III Clinical Trial Funding (Theradex Inc., Princeton, NJ), and research grant funding from NIH (NIH-R29-HL55181, NIH-U10-HL52183, NIH-M01-RR01271, NIH-R21-DK69801, NIH-K24-AT00596, NIH-UL1-RRO24146, NIH-R01-HL20985) for their generous support, without which the various clinical studies could not be carried out.

References

1. Intaglietta M. Microcirculatory basis for the design of artificial blood. Microcirculation. 1999;6:247–58.
2. Cheung ATW, Duong PL, Driessen B, Chen PCY, Jahr JS, et al. Systemic function, oxygenation and microvascular correlation during treatment of hemorrhagic shock with blood substitutes. Artif Cells Blood Substit Microcir. 2006;34:325–34.
3. Cheung ATW, To (Duong) PL, Chan DM, Ramanujam S, Barbosa MA, et al. Comparison of treatment modalities for hemorrhagic shock. Artif Cells Blood Substit Microcirc. 2007;35:175–90.
4. Chen PCY, Huang W, Stassinopoulos A, Cheung ATW. Effects of PEGylated red blood cells on microcirculation. Artif Cells Blood Substit Microcirc. 2008;36:295–309.
5. Davis E, Landau J. Clinical capillary microscopy. Charles C Thomas; 1966.

6. Colombatti R, Andemariam B. Microvasculopathy and biomarkers in sickle cell disease: the promise of non-invasive real-time *in vivo* tools. Br J Haematol. 2020. https://doi.org/10.1111/bjh.16705.
7. Sahidi M, Wanek J, Gaynes B, Wu T. Quantitative assessment of conjunctival microvascular circulation of the human eye. Springfield (Illinois) and Fort Lauderdale (Florida), USA. Microvasc Res. 2010;79(2):109–13.
8. Cheung ATW, Harmatz P, Wun T, Chen PC, Larkin EC, et al. Correlation of intracranial vessel velocity (measured by transcranial Doppler ultrasonography) with abnormal conjunctival vessel velocity (measured by computer-assisted intravital microscopy) in sickle cell disease. Blood. 2001;97:3401–4.
9. Cheung ATW, Tomic (Smith) MM, Chen PCY, Miguelino E, Li CS, et al. Correlation of microvascular abnormalities and endothelial dysfunction in Type-1 Diabetes Mellitus (T1DM): a real-time intravital microscopy study. Clin Hemorheol Microcirc. 2009;42:285–95.
10. To WJ, Telander DG, Lloyd ME, Chen PCY, Cheung ATW. Correlation of conjunctival microangiopathy with retinopathy in Type-2 diabetes mellitus (T2DM) patients. Clin Hemorheol Microcirc. 2011;47:131–41.
11. Cheung ATW, Hu BS, Wong SA, Chow J, Chan MS, et al. Microvascular abnormalities in the bulbar conjunctiva of contact lens users. Clin Hemorheol Microcirc. 2012;51:77–86.
12. Cheung ATW, Chen PCY, Wong KY, Banerjee A, Tracy BD, et al. Microvascular complications in orthokeratology (Ortho-K): a real-time study on the microvasculature in Ortho-K treatment. Clin Hemorheol Microcirc. 2019;72:119–28.
13. Smith MM, Chen PCY, Li CS, Ramanujam S, Cheung ATW. Whole blood viscosity and microvascular abnormalities in Alzheimer's disease. Clin Hemorheol Microcirc. 2009;41:229–39.
14. Chew SH, Tomic (Smith) MM, Cheung ATW. Alzheimer's disease: more than amyloid. Clin Hemorheol Microcirc. 2010;46:69–73.
15. To WJ, O'Brien VP, Banerjee A, Gutierrez AN, Li JJ, et al. Real-time studies of hypertension using non-mydriatic fundus photography and computer-assisted intravital microscopy. Clin Hemorheol Microcirc. 2012;51:1–13.
16. Cheung ATW, Chen PCY, Larkin EC, Duong PL, Ramanujam S, et al. Microvascular abnormalities in sickle cell disease: a computer-assisted intravital microscopy study. Blood. 2002;99:3999–4005.
17. Cheung ATW, Miller JW, Craig SM, To PL, Lin X, et al. Comparison of real-time microvascular abnormalities in pediatric and adult sickle cell anemia patients. Am J Hematol. 2010;85:899–901.
18. Cheung ATW, Chan MS, Ramanujam S, Rangswami A, Curl K, et al. Effects of Poloxamer 188 treatment on sickle cell vaso-occlusive crisis: computer-assisted intravital microscopy study. J Investig Med. 2004;52(6):1–5.
19. Samarron SL, Miller JW, Cheung ATW, Chen PCY, Lin X, et al. Homocysteine is associated with severity of microvasculopathy in sickle cell disease patients. Br J Haematol. 2019. https://doi.org/10.1111/bjh.16618.
20. Intaglietta M. Microcirculatory effects of hemodilution: background and analysis. In: Tuma RF, White JV, Messmer K, editors. The role of hemodilution in optimal patient care. Munchen., FRG: Zuckschwerdt W; 1989. p. 21–41.
21. Intaglietta M, Johnson PC, Winslow RM. Microvascular and tissue distribution. Cardiovasc Res. 1996;32:632–43.
22. Kerger H, Saltzman DJ, Menger MD, Messmer K, Intaglietta M. Systemic and subcutaneous microvascular pO_2 dissociation during 4-h hemorrhagic shock in conscious hamster. Am J Phys. 1996;270:H827–36.
23. Messmer K. Hemodilution. Surg Clin N Am. 1975;55:659–78.
24. Messmer K, Kreimeier U, Intaglietta M. Present state of intentional hemodilution. Eur Surg Res. 1986;18:254–63.
25. Tsai AG, Friesenecker B, Kerger H, Sakai H, Intaglietta M. The mechanism of tissue oxygenation and the design of oxygen carrying plasma expanders. In: Winslow RM, Vandegriff KD, Intaglietta M, editors. Advances in blood substitutes, industrial opportunities and medical challenges. Boston: Birkhauser; 1997. p. 189–206.
26. Tsai AG, Friesenecker B, McCarty M, Sakai H, Intaglietta M. Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skin fold model. Am J Phys. 1998;275:H2170–80.
27. Tsai AG, Kerger H, Intaglietta M. Microcirculatory consequences of blood substitutes with αα-hemoglobin. In: Winslow RM, Vandegriff KD, Intaglietta M, editors. Blood substitutes, physiological basis of efficacy. Boston: Birkhauser; 1995. p. 155–74.
28. Winslow RM. Blood substitutes. Sci Med. 1997;4:54–63.
29. Tsai AG, Kerger H, Intaglietta M. Microvascular oxygen distribution: effects due to free hemoglobin in plasma. In: Winslow RM, Vandegriff KD, Intaglietta M, editors. Blood substitutes. New challenges. Boston: Birkhauser; 1996. p. 124–31.
30. Macdonald VW, Winslow RM, Marini MA, Klinker MT. Coronary vasoconstrictor activity of purified and modified human hemoglobin. Biomater Artif Cells Artif Organs. 1990;18:263–82.
31. Cheung ATW, Driessen B, Ramanujam S, Barbosa M, Chan D, et al. Vasopressin treatment in hemorrhagic shock. Medimond Editore - Int Proc. 2004:81–5.
32. Cheung ATW, Jahr JS, Driessen B, Duong PL, Chan MS, et al. The effects of hemoglobin-based oxygen carrier (Hemoglobin Glutamer-200 Bovine) on the microcirculation in a canine hypovolemia model: a non-invasive computer-assisted intravital microscopy study. Anesth Analg. 2006;2:65–71.
33. Tsai AG, Friesenecker B, Mazzoni MC, Kerger H, Buerk DG, et al. Micovascular and tissue oxygen gradients in the rat mesentery. Proc Natl Acad Sci U S A. 1998;95:6590–5.
34. Wiggers HC, Ingraham RC, Dille J. Hemorrhagic-hypotension shock in locally anesthesized dogs. Am J Phys. 1945;143:126–33.
35. Munoz C, Aletti F, Govender K, Cabrales P, Kistler EB. Resuscitation after hemorrhagic shock in the microcirculation: targeting optimal oxygen delivery in the design of artificial blood substitutes. Blood Substit Microcirc. 2020;7:1–11. (Article 585638).
36. Kahn JM, Kress JP, Hall JB. Skin necrosis after extravasation of low dose vasopressin for septic shock. Crit Care Med. 2002;30:1899–901.
37. Laszlo F, Pavo I, Szepes Z. Deleterious action of vasopressin in gastroduodenal ulceration: experimental and clinical observations. Scand J Gastroenterol Suppl. 1998;228:62–7.
38. Jahr JS, Osgood S, Rothenberg SJ, Li Q-L, Butch AW, Gunther RA, Cheung AT, Driessen B. Lactate interference by hemoglobin-based oxygen carriers (Oxyglobin, Hemopure and Hemolink). Anesthesia and Analgesia 2005;100:431–6.
39. Osgood SL, Jahr JS, Desai P, Tsukamoto J, Driessen B. Does methemoglobin from oxidized HBOC (Hemoglobin glutamer-200) interfere with lactate measurement (YSI 2700 Biochemistry Analyzer)? Anesthesia and Analgesia 2005;100:437–9.
40. Khan AK, Jahr JS, Nesargi S, Rothenberg SJ, Tang Z, et al. Does lead interfere with hemoglobin-based oxygen carrier (HBOC) function. Anesth Analg. 2003;96:1813–20.
41. Driessen B, Zarucco L, Gunther RA, Burns PM, Lamb SV, et al. Effects of low volume Hemoglobin Glutamer-200 (bovine) versus saline and arginine vasopressin resuscitation on systemic and skeletal muscle blood flow and oxygenation in a canine hemorrhagic shock model. Crit Care Med. 2007;35:1–9.
42. To PL, Gunther RA, Jahr JS, Holtby B, Driessen B, et al. Comparison of the macro-circulatory effects of the new generation plasma expander EAF-HEXA-PEGylated albumin (EAF-PEG-BSA) in a rabbit hemorrhagic shock model: a comparison with conventional crystalloid and colloid solutions. J Anesth Clin Res. 2013;4(8). https://doi.org/10.4172/2155-6148.1000343

Nitric Oxide and Hemoglobin: Physiological Implications

9

Xinggui Shen, Alan D. Kaye, Elyse M. Cornett, and Christopher G. Kevil

Introduction

The physiological importance of NO was recognized in 1998 by the Noble Prize in Physiology or Medicine to Drs. Robert Furchgott, Louis Ignarro, and Ferid Murad for their discoveries regarding NO as a signaling molecule in the cardiovascular system. It is now well established and clear that NO bioavailability serves a central role in maintaining not only cardiovascular health but also immunity, hemostasis, cell growth, and survival, among other functions. As such, maintaining sufficient and adequate amounts of bioavailable NO is key for tissue homeostasis and overall health. Importantly, NO reacts with heme-containing proteins, including hemoglobin, to form different species, including nitrosyl hemoglobin or S-nitrosohemoglobin, which can be modulated by O_2 partial pressure. The reaction of NO/nitrosonium anion and protein thiols can also lead to nitroso thiol formation, which decomposes to generate NO. Additionally, NO can also generate reactive nitrogen species (RNS) through a diffusion-limited reaction with reactive oxygen species (ROS) such as superoxide to form peroxynitrite ($ONOO^-$).

NO is a colorless gaseous free radical with an unpaired electron making it highly reactive. NO can readily react with oxygen and water to be converted into nitrogen dioxide and nitrous acid (HNO2), respectively. HNO2 is a weak and monobasic acid with a pKa of 3.39. NO can be generated through enzymatic or non-enzymatic pathways. Under certain pathophysiological conditions, non-enzymatic generation of NO occurs through nitrite/nitrate reduction mechanisms [1]. The predominant mechanism of NO production is through the enzyme family of nitric oxide synthases (NOS), including neuronal NOS (NOS 1), inducible NOS (NOS 2), and endothelial NOS (NOS 3) [2]. The NOS enzyme is a homodimer that has N-terminal oxygenase and C-terminal reductase domains, with calmodulin (CaM) acting as the interdomain linker. Generation of NO through NOS requires several factors, including the substrate arginine, co-substrates molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH), and cofactors, including flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH4). The process of NO production via NOS oxidation-reduction reaction of L-arginine involves a series of steps, including transfer of electrons from NADPH at the reductase domain to the heme in the oxygenase domain, through FAD and FMN. The CaM domain is involved in facilitating the flow of electrons, which is enhanced by intracellular calcium (Ca^{+2}). At the oxygenase domain, electrons oxidize L-arginine to L-citrulline, and NO is produced [3]. BH4 is important to stabilize the active NOS dimer and direct the catalytic electron flow to L-arginine [4]. Separately, non-enzymatic NO generation has been shown to involve nitrate reduction to nitrite by host commensal bacteria, subsequently followed by one-electron Nitric oxide (NO): reduction of nitrite back to NO under permissive tissue conditions or via protein biochemistry (e.g., deoxyhemoglobin and xanthine oxidoreductase) [5].

Nitric oxide (NO) is also known as an endothelium-derived relaxing factor. It acts as a vasodilator, regulating

X. Shen
Department of Pathology, LSU Health Shreveport, Shreveport, LA, USA
e-mail: xshen@lsuhsc.edu

A. D. Kaye (✉)
Department of Anesthesiology and Department of Pharmacology, Toxicology, and Neurosciences, LSU School of Medicine, Shreveport, LA, USA

Department of Anesthesiology and Pharmacology, LSU School of Medicine, New Orleans, LA, USA

Department of Anesthesiology and Pharmacology, Tulane School of Medicine, New Orleans, LA, USA
e-mail: akaye@lsuhsc.edu

E. M. Cornett
Department of Anesthesiology, LSU Health Shreveport, Shreveport, LA, USA
e-mail: ecorne@lsuhsc.edu

C. G. Kevil
LSU Health Shreveport, Shreveport, LA, USA
e-mail: ckevil@lsuhsc.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_9

vascular tone, blood pressure, and hemodynamics, a role that nitrate donor therapy exploits in the treatment of angina, heart failure, pulmonary hypertension, and erectile dysfunction. Additionally, related to its potent antioxidant, anti-inflammatory, and antithrombotic properties, it is antiatherogenic and antiatherothrombotic. NO signaling regulates the contractility and metabolism of skeletal muscle and myocardium and is tightly coupled to insulin signaling. The coordination of skeletal muscle and myocardial energy demand and supply by vascular and muscle NO signaling is critical for carbohydrate and fatty acid homeostasis in the total body. NO signaling in the mitochondria is responsible for a large portion of NO's metabolic effect, which at low physiologic levels connects cellular energy demand and mitochondrial energy supply while also affecting mitochondrial oxidative stress and calcium handling. Mitochondria are also the site of potentially fatal adverse effects associated with excessive NO levels caused by inflammation. Senescence, oxidative stress, inflammation, endothelial dysfunction, vascular disease, insulin resistance, and type 2 diabetes mellitus are all characteristics of NO-deficient states. NO-enrichment therapy should be beneficial not only hemodynamically but also metabolically. In contrast, strategies for reducing excessive NO in states such as septic shock are required [6].

Nitric Oxide Cardiovascular Effects

Nitric oxide (NO) plays a role in contractility, and heart rate regulation restricts cardiac remodeling after infarction, and leads to the protective effect of ischemic pre- and postconditioning. NO at low concentrations inhibits phosphodiesterase III, stopping cAMP from being hydrolyzed. Following activation of protein kinase A, sarcolemmal voltage-operated and sarcoplasmic ryanodin receptor Ca(2+) channels are opened, increasing myocardial contractility. Increased NO levels result in increased cGMP output, which causes cardiodepression in response to protein kinase G (PKG) activation and blockade of sarcolemmal Ca(2+) channels. Additionally, NO plays a role in the decreased contractile response to adrenergic stimulus in heart failure. Inhibiting NO synthase (NOS) is clearly associated with a decrease in heart rate. Notably, if baroreceptors are activated by changes in blood pressure, the immediate influence of NOS inhibition may be changed. Finally, NO can exert a protective effect against the detrimental effects of cardiac remodeling following myocardial infarction through the cGMP pathway. NO exerts its defensive effect primarily via the guanylyl cyclase-cGMP pathway, which results in activation of PKG, which opens mitochondrial ATP-sensitive potassium channels and inhibits mitochondrial permeability transfer pores. NO formed by vascular and endocardial endothelial NOS, and neuronal and inducible synthases, acts on the muscle. Although endothelial synthase plays a significant role in the basal regulation of contractility, the inducible isoform is primarily responsible for the cardiodepression seen in septic shock [7].

Nitric Oxide Vascular Effects

Endothelial cells produce vascular NO, which causes vasodilation. NO is the most powerful endogenous vasodilator, affecting primarily conduit vessels rather than microvasculature. NO/cGMP/cGK signaling causes vasodilation by autocrine increasing NO and BH4 levels within the endothelium and paracrinely relaxing adjacent vascular smooth muscle cells (VSMCs) by (1) decreasing cytoplasmic Ca^{2+} concentrations and (2) decreasing myofibrillar Ca^{2+} sensitivity [8]. NO is involved in flow-mediated vasodilation and acts as an antagonist to vasoconstrictor effects. It works by reducing vascular stiffness and blood pressure. NO plays a critical role in blood flow regulation, vascular tone, and blood pressure regulation [9].

The endothelium is constantly damaged mechanically, chemically, and ischemically. Endothelial stem and progenitor cells (EPCs) derived from bone marrow participate in repair processes at the site of injury, normalizing endothelial function. NO protects EPCs' functional capacity for vascular repair and angiogenesis. Finally, NO inhibits platelet activation, aggregation, and adhesion to the endothelium in both cGMP-dependent and -independent ways [8].

Nitric Oxide and Hemodynamic Regulations

Nitric oxide (NO) produced by endothelial NO synthase (eNOS) is critical for coronary blood flow regulation via vasodilation and decreased vascular resistance, and platelet aggregation and adhesion inhibition, thereby preventing coronary circulatory failure, thrombosis, and atherosclerosis. When coronary perfusion is compromised, NO reduces myocardial oxygen consumption. Pathogenic factors such as smoking, excessive salt intake, obesity, aging, hypercholesterolemia, hyperglycemia, and hypertension impair endothelial function. Reduced NOS expression and activity, decreased NO bioavailability, and increased production of oxygen radicals and endogenous NOS inhibitors all contribute to endothelial dysfunction. Increased superoxide generation is mediated by NADPH oxidase, xanthine oxidase, and NOS uncoupling. Plasma levels of asymmetric dimethylarginine, an endogenous NOS inhibitor, are increased due to an inability of dimethylarginine dimethylaminohydrolase and alanine-glyoxylate aminotransferase 2 to catalyze their degradation. Reduced coronary blood flow is caused by impaired coronary arteriolar dilation induced by perivascular nitrergic nerve activation. NO derived from nNOS, either alone or in combination with eNOS, protects the myocardium from serious ischemic insults. Increased

iNOS expression caused by ischemia contributes to myocardial contractile dysfunction. Preventive and therapeutic measures, such as lifestyle modification and treatment with therapeutic agents that aim to eliminate pathogenic factors for endothelial dysfunction or nNOS-derived NO deprivation are critical for the prophylaxis and prevention of coronary artery disease [10].

Hemoglobin (Hb)

Hemoglobin is synthesized in the proerythrocyte of the bone marrow and in reticulocytes during cellular differentiation in the bone marrow. Residual hemoglobin formation can also occur in reticulocytes after denucleation due to the presence of residual RNA before entering the vasculature. First, the heme molecule is synthesized as the combination of protoporphyrin IX and iron (Fe^{+2}), which consists of four pyrrole molecules cyclically linked together with the iron ion bound in the center. Each heme molecule then combines with a long polypeptide chain called a globin, producing a hemoglobin chain. Four hemoglobin chains bind loosely together to form the whole hemoglobin molecule. There are slight differences among globin chains (such as alpha, beta, gamma) based on the amino acid sequence. In an adult human, the most common hemoglobin type is a tetramer, consisting of two α (141 amino acid residues) and two β subunits (146 amino acid residues) with a molecular weight of 64 kDa [11].

Hemoglobin can bind loosely and reversibly with oxygen. The iron ion is the site of oxygen binding, and each heme can bind one oxygen molecule. The iron ion can exist in two chemical states, including ferrous (Fe^{+2}) and ferric (Fe^{+3}). Importantly, hemoglobin binds oxygen when iron is in the ferrous state. When heme iron is oxidized to ferric, oxygen-binding cannot occur. The ferric form of hemoglobin is called methemoglobin (metHb), and metHb can be eventually reactivated to bind oxygen by reducing the iron center by methemoglobin reductase [12].

Once oxygen binds to a heme group, electrostatic forces between the individual chains relax, resulting in exposure to other oxygen-binding sites and increased affinity for oxygen. Thus, hemoglobin predominantly binds oxygen in the lungs with high-O_2 pressure and subsequently releases oxygen in the peripheral tissue that contains low-O_2 pressure. The affinity of hemoglobin to oxygen is affected by several factors that are discussed elsewhere. However, hydrogen and carbon dioxide decrease the affinity of hemoglobin to oxygen, and 2,3-diphosphoglycerate stabilizes the deoxyhemoglobin configuration, also decreasing the affinity of hemoglobin to oxygen. Due to the various reactive chemistries and since hemoglobin concentration is high in red cells, it is important that it be effectively compartmentalized by the red cell lipid bilayer membrane. This membrane protects the hemoglobin tetramers from dissociation, resulting in the formation of dimers, and monomers would ultimately be cleared by the kidney, often contributing to nephrotoxicity [13]. Moreover, an inability to compartmentalize hemoglobin decreases oxygen binding and exchange efficiencies while also allowing secondary oxidation-reduction reactions to occur that could damage cells and tissues.

Interactions Between NO and Hemoglobin

The rate at which hemoglobin reacts with nitric oxide (NO) is limited by the speed of the diffusion of NO into the heme pocket. The reaction results in the formation of nitrate when hemoglobin is oxygenated. This is referred to as the dioxygenation reaction. Because nitrate is biologically inert at the concentrations reached during the dioxygenation reaction, the only role hemoglobin was once thought to play in NO signaling was to inhibit it. However, several mechanisms by which hemoglobin can preserve, control, and even generate NO activity have been discovered. These mechanisms entail the compartmentalization of reacting species and the conversion of NO from or into other NO-transporting species such as nitrosothiols or nitrite. Despite many research studies in this area, fundamental questions remain regarding the precise mechanisms of NO activity preservation, and whether and how Hb generates NO activity.

Nitrosyl Hemoglobin

Hemoglobin only binds oxygen when the iron heme is ferrous, forming oxyhemoglobin. NO can also bind to the ferrous heme, forming iron nitrosyl hemoglobin, including alpha-nitrosyl and beta-nitrosyl hemoglobin. Electron paramagnetic resonance (EPR) is typically used to distinguish these two species. For alpha-nitrosyl heme, the bond between the histidine and iron breaks, so the heme is pentacoordinate (one bound to the NO and four to the heme porphyrin ring). The affinity of NO for ferrous heme is very significant with a dissociation constant (Kd) of 10^{-10}~10^{-11} M [14]., meaning that hemoglobin likely serves as a biochemical 'sink' for NO in the blood, especially given the concentration of hemoglobin in erythrocytes. Nitric oxide can also bind to the ferric hem, but at a much lower affinity ($Kd = 2.5 \times 10^{-4}$ M). Lastly, NO-mediated nitroso thiol formation occurs at cysteine residues (cys93) of the β subunit resulting in the formation of S-nitroshemoglobin (SNO-Hb).

NO Bioactivity and Hemoglobin

NO can react with oxyhemoglobin (oxyHb) to form methemoglobin and nitrate, and it occurs at a reaction rate of $6–8 \times 10^{-7}\ M^{-1}\ S^{-1}$ [15–17]. Importantly, this reaction is rate limited primarily by diffusion of NO to the heme pocket [18, 19]. With 10 mM Heme in the blood, the half-life of NO under oxygenated conditions would be predicted to be ~1 μs. During this time, NO could diffuse about 0.1 μm (assuming a diffusion constant of 3000 $\mu m^2\ s^{-1}$) so that any NO that diffuses into the blood and encounters oxyHb would be rap-

idly converted to nitrate [20]. The amount of nitrate in plasma is typically tens of micromolar [21], while NO is present in the nanomolar range or less [22]. Thus, conversion of NO to nitrate does not significantly increase the concentration of plasma nitrate.

NO can also bind to deoxygenated ferrous hemoglobin. In principle, NO can dissociate from the heme. The dissociation rate constant of NO from heme are 1×10^{-3} s^{-1} for T-state beta hemes, 4×10^{-4} s^{-1} for T-state alpha hemes, and 1×10^{-5} s^{-1} for R-state, respectively [23–25]. Hence, heme nitrosylation does not permanently remove NO from biological NO signaling pools.

S-nitrosothiols are potentially an effective transporter of NO bioactivity through the ultimate release of bound NO or mimic NO [26, 27]. Although this reaction is much slower than that of NO and oxygenated or deoxygenated Hb [28], the NO^+ moiety can be transferred among thiol groups, resulting in the formation of SNO-Hb. It is a very effective way of transducing NO bioactivity [29]. Stamler and his colleagues have shown this intriguing mechanism [30–32]. The central component of this mechanism is that iron nitrosyl Hb is formed in the tissues, and NO is transferred to β93 cysteine residue to generate SNO-Hb during T to R transition [31].

NO, Red Cell Biology and Blood Substitutes

As discussed above, NO is important for both tissue function and health while also highly chemically reactive with heme-containing proteins such as hemoglobin. Thus, it is now clear that blood substitute approaches may mediate pathological side effects through decreasing NO levels and or functions [33]. Cell-free, cross-linked hemoglobin agents have notoriously depleted NO leading to cardiovascular complications. LEH substitutes have shown some promise in attenuating this problem due to hemoglobin containment within a lipid bilayer. Although this approach has shown some promise, the ability to recapitulate the spectrum of NO-Hb biochemical interactions that could enhance tissue oxygenation have yet to be realized.

NO Scavenging

Numerous discoveries have been made through in vitro studies of aged, diseased RBCs and naturally occurring mutant Hbs, and through animal and human studies. As a result, alternative direct intervention strategies for preventing heme-mediated Hb oxidative toxicity have evolved to the point where it is feasible to include antioxidant scavenging proteins and iron-reducing reagents in HBOCs to retard Hb-damaging prooxidant reactions and preserve Hb function. For instance, glutathione (GSH), a critical scavenger of free radicals and a potent endogenous antioxidant that aids in the protection of RBCs against oxidative injury, has been crosslinked to Hb. Hemopexin (HPX) is an acute-phase plasma protein found in concentrations ranging from 8 to 21 mM in circulation. By transporting plasma heme to the liver, HPX acts as a specific scavenger of plasma heme and clearance, and, like Hp, HPX acts as a critical plasma protector against Hb oxidation by-products. HPX has the tightest binding kinetics when compared to other plasma heme scavengers (such as albumin, high- and low-density lipoproteins, and a1-microglobulin) [34].

Conclusion

In summary, NO and hemoglobin engage in important biochemical functions necessary for oxygen delivery and carbon dioxide removal to match local metabolic demand within tissues and organs. However, many questions remain surrounding the precise mechanisms of NO chemical biology in red blood cells and for their preservation, and how hemoglobin alters NO functions in acute and chronic trauma or disease settings making Hb-NO research an important and exciting area for further research. In this regard, NO will continue to be a major factor in the success of Hb carrying products in the future.

Key Points

- The rate of hemoglobin reactivity with nitric oxide (NO) is constrained by the rate of NO diffusion into the heme pocket.
- When hemoglobin is oxygenated, a chemical reaction occurs that produces nitrate.
- Since nitrate is biologically inert at the amounts achieved during the dioxygenation reaction, hemoglobin was previously believed to have just an inhibitory function in NO signaling.
- Several pathways, however, have been discovered by which hemoglobin can maintain, regulate, and even produce NO action.
- These pathways include the compartmentalization of reacting species and the transfer of NO to or from other NO-transporting species such as nitrosothiols or nitrite.
- Despite numerous research studies, basic concerns about the exact processes of NO activity protection, and when and how Hb produces NO activity, exist.

References

1. Kevil CG, Kolluru GK, Pattillo CB, Giordano T. Inorganic nitrite therapy: historical perspective and future directions. Free Radic Biol Med. 2011;51(3):576–93.
2. Förstermann U, Münzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation. 2006;113(13):1708–14.

3. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J. 2012;33(7):829–37, 837a–837d.
4. Tzeng E, Billiar TR, Robbins PD, Loftus M, Stuehr DJ. Expression of human inducible nitric oxide synthase in a tetrahydrobiopterin (H4B)-deficient cell line: H4B promotes assembly of enzyme subunits into an active dimer. Proc Natl Acad Sci U S A. 1995;92(25):11771–5.
5. Bir SC, Kolluru GK, McCarthy P, Shen X, Pardue S, Pattillo CB, et al. Hydrogen sulfide stimulates ischemic vascular remodeling through nitric oxide synthase and nitrite reduction activity regulating hypoxia-inducible factor-1α and vascular endothelial growth factor-dependent angiogenesis. J Am Heart Assoc. 2012;1(5):e004093.
6. Levine AB, Punihaole D, Levine TB. Characterization of the role of nitric oxide and its clinical applications. Cardiology. 2012;122(1):55–68.
7. Rastaldo R, Pagliaro P, Cappello S, Penna C, Mancardi D, Westerhof N, et al. Nitric oxide and cardiac function. Life Sci. 2007;81(10):779–93.
8. Münzel T, Feil R, Mülsch A, Lohmann SM, Hofmann F, Walter U. Physiology and pathophysiology of vascular signaling controlled by cyclic guanosine 3′,5′-cyclic monophosphate–dependent protein kinase. Circulation. 2003;108(18):2172–83.
9. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, et al. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. Nat Med. 2003;9(11):1370–6.
10. Toda N, Toda H. Coronary hemodynamic regulation by nitric oxide in experimental animals: recent advances. Eur J Pharmacol. 2011;667(1–3):41–9.
11. Van Beekvelt MC, Colier WN, Wevers RA, Van Engelen BG. Performance of near-infrared spectroscopy in measuring local O(2) consumption and blood flow in skeletal muscle. J Appl Physiol (1985). 2001;90(2):511–9.
12. Linberg R, Conover CD, Shum KL, Shorr RG. Hemoglobin based oxygen carriers: how much methemoglobin is too much? Artif Cells Blood Substit Immobil Biotechnol. 1998;26(2):133–48.
13. Stowell CP, Levin J, Spiess BD, Winslow RM. Progress in the development of RBC substitutes. Transfusion. 2001;41(2):287–99.
14. Cooper CE. Nitric oxide and iron proteins. Biochim Biophys Acta. 1999;1411(2):290–309.
15. Doyle MP, Hoekstra JW. Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. J Inorg Biochem. 1981;14(4):351–8.
16. Eich RF, Li T, Lemon DD, Doherty DH, Curry SR, Aitken JF, et al. Mechanism of NO-induced oxidation of myoglobin and hemoglobin. Biochemistry. 1996;35(22):6976–83.
17. Herold S, Exner M, Nauser T. Kinetic and mechanistic studies of the NO*-mediated oxidation of oxymyoglobin and oxyhemoglobin. Biochemistry. 2001;40(11):3385–95.
18. Olson JS, Rohlfs RJ, Gibson QH. Ligand recombination to the alpha and beta subunits of human hemoglobin. J Biol Chem. 1987;262(27):12930–8.
19. Olson JS, Phillips GN. Kinetic pathways and barriers for ligand binding to myoglobin. J Biol Chem. 1996;271(30):17593–6.
20. Helms C, Kim-Shapiro DB. Hemoglobin-mediated nitric oxide signaling. Free Radic Biol Med. 2013;61:464–72.
21. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov. 2008;7(2):156–67.
22. Hall CN, Garthwaite J. What is the real physiological NO concentration in vivo? Nitric Oxide. 2009;21(2):92–103.
23. Moore EG, Gibson QH. Cooperativity in the dissociation of nitric oxide from hemoglobin. J Biol Chem. 1976;251(9):2788–94.
24. Azizi F, Kielbasa JE, Adeyiga AM, Maree RD, Frazier M, Yakubu M, et al. Rates of nitric oxide dissociation from hemoglobin. Free Radic Biol Med. 2005;39(2):145–51.
25. Sharma VS, Ranney HM. The dissociation of NO from nitrosylhemoglobin. J Biol Chem. 1978;253(18):6467–72.
26. Zhang Y, Hogg N. S-Nitrosothiols: cellular formation and transport. Free Radic Biol Med. 2005;38(7):831–8.
27. Hogg N. Biological chemistry and clinical potential of S-nitrosothiols. Free Radic Biol Med. 2000;28(10):1478–86.
28. Spencer NY, Patel NK, Keszler A, Hogg N. Oxidation and nitrosylation of oxyhemoglobin by S-nitrosoglutathione via nitroxyl anion. Free Radic Biol Med. 2003;35(11):1515–26.
29. Hogg N. The biochemistry and physiology of S-nitrosothiols. Annu Rev Pharmacol Toxicol. 2002;42:585–600.
30. Pawloski JR, Hess DT, Stamler JS. Export by red blood cells of nitric oxide bioactivity. Nature. 2001;409(6820):622–6.
31. McMahon TJ, Moon RE, Luschinger BP, Carraway MS, Stone AE, Stolp BW, et al. Nitric oxide in the human respiratory cycle. Nat Med. 2002;8(7):711–7.
32. Singel DJ, Stamler JS. Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin. Annu Rev Physiol. 2005;67:99–145.
33. Cabrales P, Friedman JM. HBOC vasoactivity: interplay between nitric oxide scavenging and capacity to generate bioactive nitric oxide species. Antioxid Redox Signal. 2013;18(17):2284–97.
34. Alayash AI. Mechanisms of toxicity and modulation of hemoglobin-based oxygen carriers. Shock. 2019;52(1 Suppl):41–9.

A Brief History of the Development of Nanobiotechnology-Based Blood Substitutes

10

Thomas Ming Swi Chang

Introduction

Extensive research was carried out around the world on different types of nanobiotechnology based blood substitutes over the last 60 years [1–31, 33–38, 40–49, 51–62, 64–75, 77–100, 102–125]. Numerous groups have contributed extensively and importantly to this area. This is such a large area that it is only possible for this chapter to cover a few of the highlights. Many chapters in this book contain much more complete details.

Hemolysate and Stroma Free Hemoglobin

As early as 1937 Amberson [7] attempted to use hemolysate, the hemolyzed content of red blood cells (RBC) including the RBC membrane stroma (Fig. 10.1). In 1967 Rabiner [85] removed the membrane stroma from hemolysate to form stroma-free hemoglobin (SFHb) to prevent renal toxicity (Fig. 10.1). Despite the terminology, stroma-free hemoglobin contained variable amounts of active RBC enzymes.

In 1978, Savitsky [93] carried out preliminary clinical trial in patients and showed that SFHb still caused nephrotoxicity and cardiovascular adverse effects. The tetrameric hemoglobin molecules caused much of the problem and was made worse since once infused the tetramer broke down into even more toxic dimers. We now also know that free hemoglobin would have other problems.

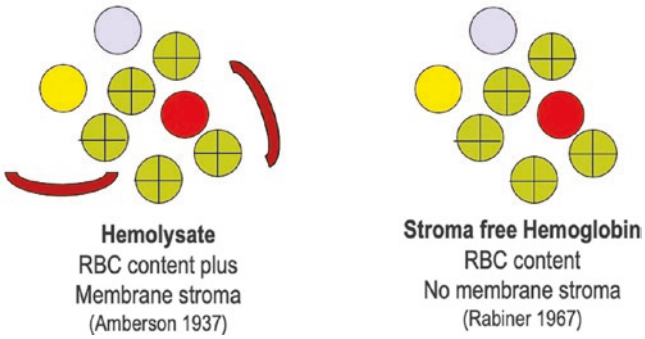


Fig. 10.1 Left: Hemolysate. Right: Stroma-free hemoglobin that contains hemoglobin and variable amounts of RBC enzymes

Early Basic Research on Nanobiotechnology Based RBC Substitute

In 1957 Chang [18] prepared the first artificial red blood cells that contain hemoglobin and red blood cell enzymes (Fig. 10.2). This had oxygen dissociation curve like that of RBC [18] Hemoglobin stayed inside as tetramers and red blood cell enzymes like carbonic anhydrase and catalase retained their activities [19, 30]. These artificial red blood cells did not have blood group antigens on the membrane and therefore do no aggregation in the presence of blood group antibodies [22]. However, the single major problem was the rapid removal of these micro-dimension artificial cells of even down to 1 micron dimension (1000 nm) from the circulation [22]. He therefore investigated nanobiotechnology based complex in the form of polyhemoglobin and conjugated hemoglobin [19–21] (Fig. 10.2).

Polyhemoglobin Based on Nanobiotechnology

In 1964 Chang [19] first use bifunctional agents to cross-link the reactive amino groups of Hb to assemble Hb molecules together into PolyHb (Fig. 10.2). The first bifunctional agent used was sebacyl chloride [19].

T. M. S. Chang (✉)
Artificial Cells and Organs Research Centre, Departments of Physiology, Medicine and Biomedical Engineering, Faculty of Medicine, McGill University, Montreal, QC, Canada
e-mail: Thomas.chang@mcgill.ca; http://www.artcell.mcgill.ca/

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_10

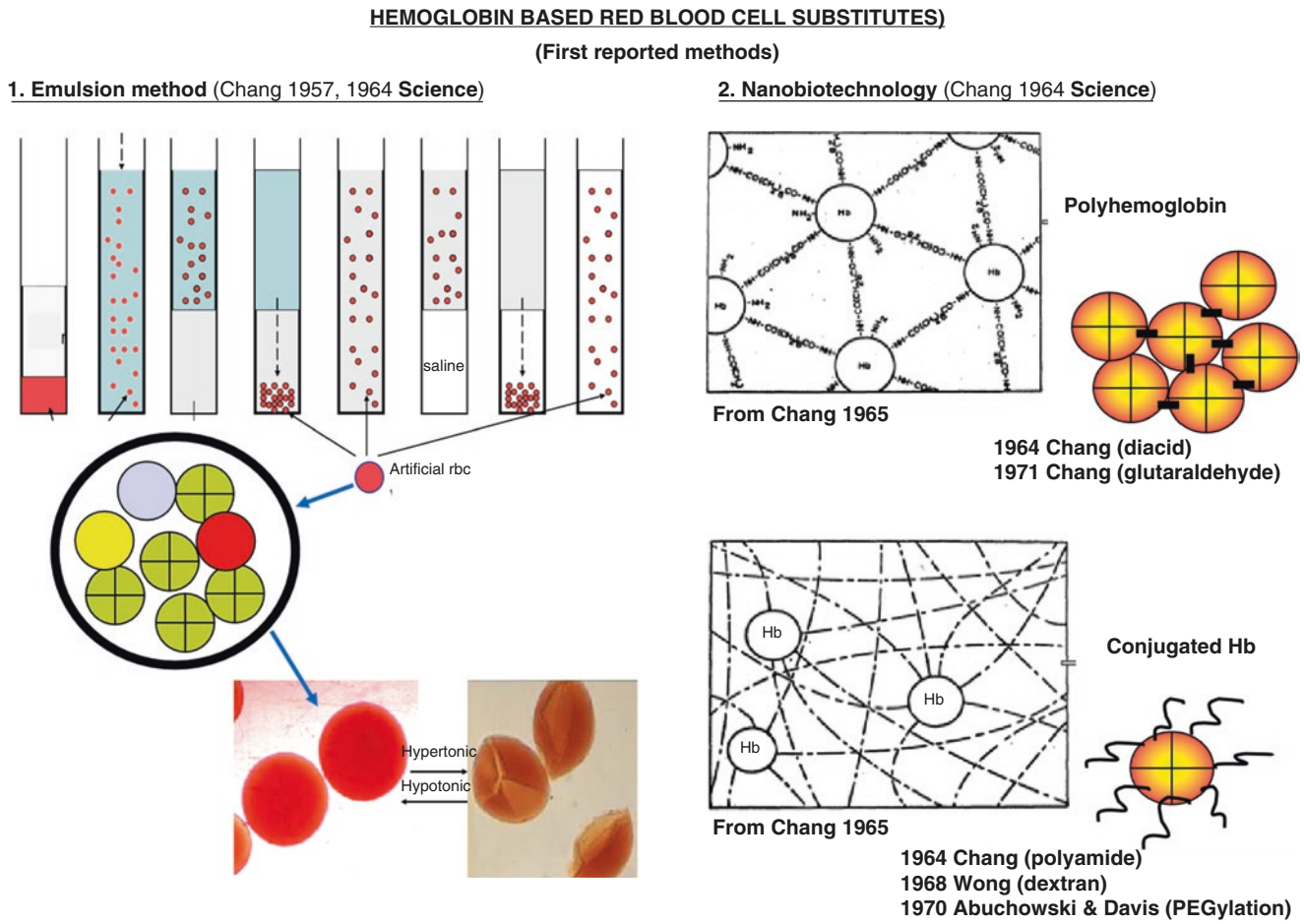


Fig. 10.2 **Upper Left** Preparation of Artificial red blood cells by emulsion. **Lower Left:** Artificial red blood cells of micro dimensions that can reversibly "crenate" in hypertonic/hypotonic solutions. **Upper Right**: Polyhemoglobin. **Lower right**: Conjugated hemoglobin. (Updated from Chang [20, 26] with copyright permission)

$$\underset{\textit{Sebacyl Chloride}}{Cl-CO-(CH_2)_8-CO-Cl} + \underset{\textit{Hemoglobin}}{HB-NH_2} = \underset{\textit{Cross-linked polyHb (Chang 1964)}}{HB-NH-CO-(CH_2)_8-CO-NH-HB}$$

The first use of another bifunctional agent, glutaraldehyde by Chang was in 1971 to cross-link hemoglobin(10gm/dl) and trace amount of catalase [21]. The reaction was as follows:

$$\underset{\textit{Glutaraldehyde}}{H-CO-(CH_2)_3-CO-H} + \underset{\textit{hemoglobin}}{HB-NH_2} = \underset{\textit{Cross-linked PolyHb (Chang, 1971)}}{HB-NH-CO-(CH_2)_3-CO-NH-HB}$$

In the 1971 glutaraldehyde procedure, cross-linking could be adjusted so that the crosslinked PolyHb could be in a soluble state [21]. This resulted in less steric hindrance and greater ease of substrate diffusion. This cross-linked hemoglobin molecules to one another (intermolecular). It could also cross-link the hemoglobin internally (intramolecular). This principle was later developed by many groups around the world.

First Generation Hemoglobin Based Oxygen Carriers (HBOCs)

Four General Types of HBOCs

The four types of most studied first-generation hemoglobin-based oxygen carriers are shown in Fig. 10.3. These are polyhemoglobin, conjugated hemoglobin, intramolecu-

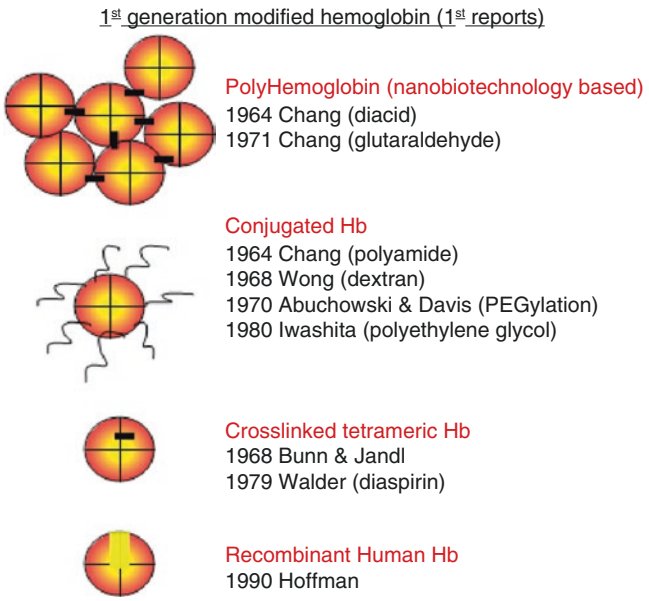


Fig. 10.3 The four types of most studied first-generation hemoglobin-based oxygen carriers

larly crosslinked tetrameric hemoglobin and recombinant hemoglobin

Development During the HIV Contaminated Donor Blood Crisis

As seen from Fig. 10.3, most of the basic principles for modified Hb were already available by the 1960's. Unfortunately, there was no public demand or interest on these approaches. Thus, only a few investigators were doing research on nanobiotechnological approaches for modified Hb. This effort is suddenly intensified at the end of the 1980s, when HIV contaminated donor blood became a major public crisis. As a result, extensive studies and extensions were belatedly carried out by many groups around the world. (Table 10.1)

Red blood cells have three major functions: (1) transport oxygen (2) remove damaging oxygen radicals and (3) transport carbon dioxide CO_2. The urgency of H.I.V. in donor blood necessitated the development of the simplest system in the shortest time in the form of oxygen carriers.

Intramolecularly Crosslinked Hemoglobin

Initially, the most extensive effort was Baxter's development of intramolecularly crosslinked tetrameric hemoglobin [15, 16, 84, 105]. Vasoconstriction in clinical trials led to the proposal that the intercellular junctions of the endothelial lining of vascular wall allowed tetrameric Hb to enter the interstitial space. There, Hb acted as a sink in binding and removing

Table 10.1 H.I.V. contaminated donor blood crisis and active groups

POLYHb, CONJUGATED Hb & XLINKED TETRAMERIC Hb
Acharya, Agishi, Alayash, Bakker, Baldwin, Blumenstein, Benesch, Biro, Bonhard, Bucci, Burhop, Chang, D'Agnillo, DeAngello, DeVenuto, DeWoskin, Estep, Fagrell, Faivre, Feola, Fratantoni, Gawryl, Gould, Greenburg, Gulati, Hess, Hori, Hsia, Hughes, Intaglietta, Iwashita, Jacobs, Jesch, Keipert, Klugger, Liu, Lowe, MacDonald, Magnin, Manning, Meinert, Messmer, McKenzie, Moss, Nelson, Nose, Panter, Pearce, Powanda, Privalle, Przybelski, Pluira, Rausch, Seetharama, Sehgal, Sekiguchi, Sideman, Shorr, Su, Tsai,Valeri, Vandegriff, Winslow, Wong, Yang and many others
Recombinant and transgenic hemoglobin Casparl, Fronticelli, Hoffman, Kumar, Lemon, Looker, Olson, Shih, Shoemaker, Stetler and many others
Encapsulated Hb (Artificial RBC)
Beissinger, Chang, Djordjevich, Farmer, Feuerstein, Gaber, Goin, Horinouchi, Hunt, Ikeda, Kondo, Kobayashi, Lee, Lutz, Miller, Nishiya, Ogata, Powanda, Phillips, Rabinovic, Rudolph, Sakai, Schmidt, Snohara, Su, Szebeni, Takahasi, Takaori, Takeoka, Tsuchida, Usuba, Yu and many others

nitric oxide needed for maintaining the normal tone of smooth muscles. This resulted in the constriction of blood vessels and other smooth muscles especially those of the esophagus and the GI tract. However, this could be avoided if nitric oxide removal was prevented by a modified form of stabilized intramolecularly crosslinked Bovine Hb [116] or by the inhalation of nitric oxide [119–121].

Polyhemoglobin

The 1971 basic principle of glutaraldehyde crosslinked polyhemoglobin (PolyHb) [21] was independently developed most extensively by centers around the world. Some examples of the earlier ones included Dudziak & Bonhard [40] 1980, DeVent & Zegna [37] 1982, Keipert & Chang [57] 1982, Feola et al. [44] 1983, Moss & Gould's group [46, 74] 1988, Biopure group including Wong and Rausch, Greenburg and Kim [47] Pearce & Gawryl [80], Jahr et al. [55]. There were also the use of other types of crosslinkers like o-raffinose by Hsia [52] and Bucci's OxyVita® Hb [13, 113] The following were two important key examples.

Glutaraldehyde Crosslinked Human PolyHb

The group led by Moss and Gould started the Northfield Laboratory to develop glutaraldehyde crosslinked human PolyHb [46, 74]. Their clinical trial on 171 patients shows that this product can successfully replace extensive blood loss in trauma surgery by maintaining the Hb level at the 8–10 g/dl needed for safe surgery with no reported side effects [74].

In 2009 Moore et al. reported their multicenter randomized clinical trial on pre-hospital ambulance patients [73]. Since no typing and cross-matching was needed, it could be

Table 10.2 PolyHeme clinical trial

Human Polymerized Hemoglobin for the Treatment of Hemorrhagic Shock when Blood Is Unavailable: The USA Multicenter Trial. Moore et al. (2009) J Am Coll Surg. 208: 1–13	
Ambulance.	**Hospital**
PolyHeme Group.	Do not need blood for 14 hours
Control saline Group:	Need blood in ½ hour
(See text for discussion of risk/benefit)	

used right on the spot. Their result in about 700 hemorrhagic shock patients showed that PolyHb could maintain the patients for 14 hours after reaching the hospital without the need for donor blood (Table 10.2). In the saline control group, most of the patients needed blood transfusion shortly after reaching the hospital.

There was nonfatal myocardial infarction in 3% of the PolyHeme group as compared to 0.6% in the control saline group. Risk/benefit analysis: As stated in the title, the author's objective was to investigate the use of PolyHeme when donor blood was not available. Based on the result of their study, if donor blood was not available, many patients in the control group would have died. In this situation the benefit far outweighed the risk of 3% nonfatal myocardial infarction. On the other hand, when donor blood was available it would be less risky than using PolyHeme, unless there were potential problems of H.I.V. or other contamination of the donor blood.

Glutaraldehyde Crosslinked Bovine PolyHb

In 1983, Feola's group studied the use of bovine Hb, instead of outdated human donor blood, to prepare PolyHb [44]. Bing Wong and Carl Rausch prepared ultrapure bovine hemoglobin for glutaraldehyde crosslinked bovine PolyHb and cofounded Biopure. The result of the development and clinical trials were published in several papers including those by Pearce & Gawryl [80], Greenburg and Kim [47], Jahr et al. [54, 55]. Mer et al. [72]. In one study, they carried out multicenter, multinational, randomized, single-blind, RBC-controlled Phase III clinical trials in patients undergoing elective orthopedic surgery (Table 10.3).

A total of 688 patients were randomized 1:1 to receive either the PolyHb or RBC at the time of the first perioperative RBC transfusion decision and 59.4% of the patients receiving polyHb required no RBC transfusion all the way to follow up and 96.3% avoided transfusion with RBC on the first postoperative day and up to 70.3% avoided RBC transfusion up to day 7 after. South Africa and Russia approved this for routine clinical uses in patients. Mer et al. [72] discusses Hemoglobin glutamer-250 (bovine) in South Africa consensus usage guidelines from clinician experts who have treated patients. Jahr gave a recent overall discussion of the area [54].

Table 10.3 Biopure clinical trial

PolyHemoglobin glutamer-250 (bovine)
Phase III clinical trials in 688 elective orthopedic surgery patients.
Randomized 1:1 for either the PolyHb or RBC
96.3% avoided RBC first postoperative day
70.3% avoided RBC to day 7 59.4% avoided RBC all the way
2002 South Africa & Russia approved this to avoid the use of HIV contaminated donor blood
Thus, the original aim to avoid the use of HIV donor blood has been successfully reached
References:
Pearce & Gawryl, 2006, Jahr et al. 2008, Greenburg et al. 2008, Mer et al. 2016

Conjugated Hemoglobin

In 1964 Chang showed that in the presence of diamine, sebacyl chloride crosslinks hemoglobin with polyamide to form conjugated hemoglobin [19, 20] (Fig. 10.2). In 1988 Wong crosslinked single hemoglobin molecule to a soluble dextran [99, 114]. In 1977, Abuchowski et al. prepared polyethyleneglyco-hemoglobin (PEG-Hb) [2]. This PEG approach was followed by others in Japan [53], USA [3, 96, 112], China [67] and elsewhere. Two key groups included Winslow and Keipert's group of Sangart [112] and Liu's group in China [63, 67]. Other groups who combined this with antioxidant function will be discussed in that section.

Nitric Oxide

Nitric oxide plays an important role in preventing vasoconstriction. Hemoglobin has high affinity in binding nitric oxide and is one of the causes of vasoconstriction.

Inhalation of Nitric Oxide to Prevent Vasoconstriction

Yu and Zapol [119, 120] showed in awake mice that infusion of HBOC-201 (Hemopure, OPK Biotech) induced systemic hypertension, which can be prevented by breathing NO. In awake lambs, pretreatment with inhaled NO prevented systemic and pulmonary hypertension induced by subsequent infusion of HBOC-201, Mice with endothelial dysfunction but not healthy wild-type mice, experienced vasoconstriction during transfusion of PolyHeme. This can be prevented by inhaled NO. In awake lambs, infusion of PolyHeme induces pulmonary hypertension, that could be prevented by breathing NO before infusion, followed by a low dose of NO during transfusion.

Pyridoxalated Hemoglobin Polyoxyethylene as NO Scavenger in Distributive Shock

Apex Bioscience used this in their multicenter, randomized, placebo-controlled phase III study in distributive shock

[104]. They compared the effectiveness and safety of the hemoglobin-based nitric oxide scavenger against placebo. Patients were randomized to receive 0.25 mL/kg/hr. pyridoxalated hemoglobin polyoxyethylene (20 mg Hb/kg/hr) or an equal volume of placebo, infused for up to 150 hours, in addition to conventional vasopressor therapy. The study was stopped after interim analysis.

Other Sources of Hemoglobin

In addition to hemoglobin from outdated human donor blood, Feola showed that bovine Hb could be another source [44]. Other sources included, for example, porcine Hb by the group of Zhu and Chen [123, 124], Yang's group started the use of Hb from human placental blood [64].

Zal's group [88, 121] prepared a novel natural oxygen carrier extracted from *Arenicola Marina* that had high oxygen carrying capabilities coupled with unique antioxidant properties. Another important source is the use of recombinant method.

Bioengineering and Recombinant Methods

In 1990, Hoffman et al. [51] first reported the expression of fully functional tetrameric human hemoglobin in Escherichia coli. This was followed by Looker et al. [69] and Shoemaker et al. [95] In order to prevent the removal of nitric oxide, Doherty et al. [39] used a specially designed recombinant Hb.

More recently, Bülow's group [55] carried out extensive research on protein engineering for hemoglobin-based oxygen carriers as an alternative to chemical approaches. They used sitedirected mutagenesis and gene fusion to optimize the behavior of blood substitutes. They looked at fetal hemoglobin (HbF) which has several advantages over the adult hemoglobin. They used the XTEN technology to modify Hb genetically. This XTEN polymer was genetically linked to fusion fetal hemoglobin (fHbF), and used as an alternative to PEG. They also investigated a green alternative based on plant Hbs. Compared to human Hbs, plant Hbs had high stability, low autoxidation and low heme-loss. For its production, the heterologous expression of plant Hbs in E. coli was 30–40% higher compared to human Hb.

General Discussions

Those polyhemoglobin or conjugated Hb that contained high levels of uncrosslinked population including endothelial dysfunction, oxygen radicals etc. There were great variations in the preparation of different types of HBOCs. Thus, one cannot attempt to combine the clinical trial results of different types of hemoglobin-based oxygen carriers and different clinical trials into a single meta-analysis [77].

Nanobiotechnology Based Oxygen Carriers with Antioxidant Functions

Introduction

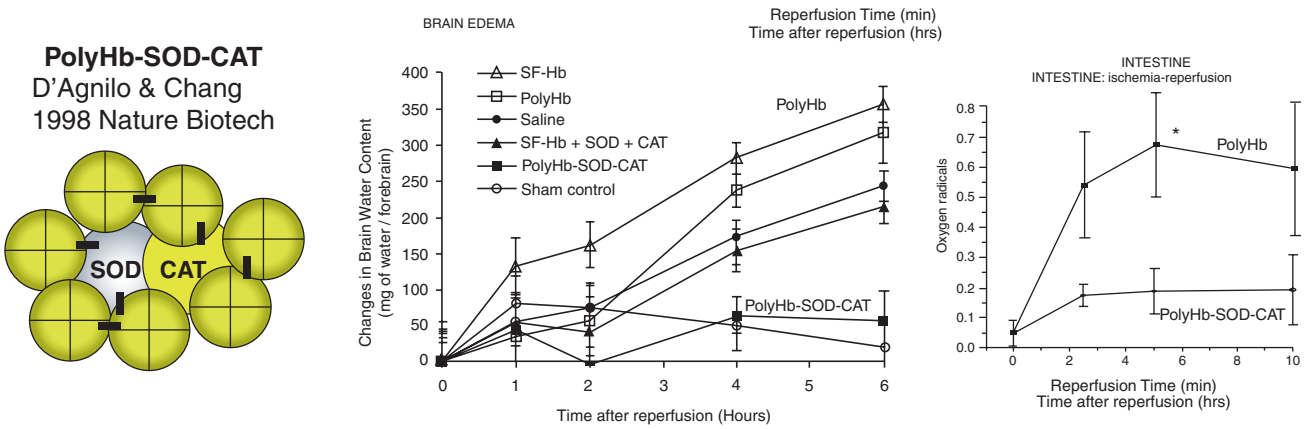
The H.I.V. contaminated donor blood crisis and the urgency for blood substitutes had led to the development of only hemoglobin-based oxygen carriers. Some even used only ultrapure hemoglobin for this. This was effective for those clinical condition that only required oxygen supply, as in elective orthopedic surgery. In arterial obstruction resulting in stroke and heart attack. Red blood cells, being 7–8 microns in diameter, would have difficulty flowing through partially obstructed vessels to supply the needed oxygen. HBOC, being a solution, could perfuse through to supply the needed oxygen. However, in ischemia, reperfusion with an oxygen carrier alone could release damaging oxygen radicals resulting in ischemia reperfusion injuries [4, 5].

PolyHb-CAT-SOD

In the early 1990s D'Agnillo and Chang prepared a soluble complex of Polyhemoglobin crosslinked to antioxidant enzymes, catalase and superoxide dismutase (PolyHb-SOD-CAT) [35]. It had the dual function of an oxygen carrier that can also remove oxygen radicals (Fig. 10.4). In this form the SOD and CAT could be enhanced to be much higher than those in red blood cells.

After 90 min of combined hemorrhagic shock and brain ischemia in rats, reinfusion of PolyHb-SOD-CAT did not cause brain edema (Fig. 10.4) [83]. On the other hand, PolyHb or a solution contain free Hb, SOD and CAT caused significant increases in brain edema (Fig. 10.4). Reperfusion with PolyHb resulted in significant increase in the breakdown of the blood-brain barrier and an increase in brain water (brain edema). On the other hand, PolyHb-SOD-CAT did not result in these adverse changes (Fig. 10.4).

Ischemia-reperfusion injury in severe sustained hemorrhagic shock could result in damage to the intestine with leakage of E-coli or endotoxin to the systemic circulation resulting in irreversible shock. Thus, we studied the ex vivo perfusion of isolated rat small intestine in a model of intestinal ischemia reperfusion [86]. We reported in 1997 that ischemic small intestine released damaging oxygen radicals when reperfused with PolyHb. However, PolyHb-SOD-CAT reperfusion did not increase oxygen radical release. This could be important during intestinal surgery or organ storage



Examples of other approaches:

- **Hsia's group** Conjuated hemoglobin with synthetic antioxidant based on the convalently binding of nitroxides (Buehler et al 2004, Ma and Hsia, 2013).
- **Yang's group:** The reduction of human cord blood methemoglobin by vitamin C
- **Alayash's group,** 2004 a Hb-haptoglobin compelx can also be used to protect against oxidative stress
- **Zal's group** Arenicola marina Hb (Rousselot et al 2006) with antioxidant activity.
- **Simoni's group** (1997) added a solution with antioxidant function to their modified Hb.
- **Abuchowski's group** (2017) PEGylated bovine hemoglobin with dual carbon monoxide and oxygen delivery agent with anti-inflammatory and anti-vasoconstrictive properties
- **Others**

Fig. 10.4 **Upper left:** PolyHb-SOD-CAT D'Agnillo & Chang 1998) **Upper middle:** Unlike PolyHb, reinfusion of PolyHb-SOD-CAT did not cause brain edema in rat brain ischemia. **Upper right:** Unlike PolyHb, PolyHb-SOD-CAT reperfusion in ischemic small intestine did not release damaging oxygen radicals. (From Chang 2007 with copyright permission). Lower: Examples of many other approaches based on the above principle

for transplantation [86] Others have used this for kidney [32] and pancreatic beta cells in rats [76].

Convalently Binding of Nitroxides to PEG-Hb

Hsia's group extended the PolyHb-SOD-CAT approach to prepare a pegylated hemoglobin with synthetic antioxidant based on the convalently binding of nitroxides [14, 70]. Polynitrxylation confers superoxide dismutase mimetic activity to pegylated hemoglobin. They tested this polynitroxylated pegylated hemoglobin (PNPH aka SanFlow) for the Treatment of Hemorrhagic shock and Ischemic Stroke In a hemorrhagic traumatic brain injury (TBI) model after combined traumatic brain injury and hypotension in mice. They also tested this in a stroke model following transient occlusion of the middle cerebral artery. Transfusion of polynitroxylated pegylated hemoglobin could better maintain oxygen delivery during ischemia through the collateral circulation before reperfusion is established; could provide neuroprotection after reperfusion. They showed that small volume of 1/10th of shed blood volume of SanFlow, in conjunction with crystalloid, was could restore mean arterial pressure (MAP) to a more stable and higher level than fresh whole shed blood in a mouse model of traumatic brain injury (TBI) with hemorrhagic shock (HS). SanFlow was shown to maintain cerebral perfusion pressure (CPP) of brain to provided critical oxygen delivery to serve neurological functional needs without resulting in increased intracranial pressure (ICP) and without cerebral edema of the mouse brain.

Dual Carbon Monoxide and Oxygen Delivery Agent

Abuchowski's group [1] reported a dual carbon monoxide and oxygen delivery agent with antiinflammatory and antivasoconstrictive properties from carbon monoxide, improved rheology from polyethylene glycol, and oxygen delivery specifically to hypoxic tissues from PEGylated bovine hemoglobin. They carried out preclinical and clinical studies that showed safety with evidence of efficacy. Animal models of hemorrhagic shock and focal cerebral ischemia displayed the ability of SANGUINATE® to down-regulate inflammatory markers, deliver oxygen, and improve survival. Early clinical trials in patients exposed to either single or multiple doses there was no meaningful adverse effects. Signs of efficacy include improved neurological status, reduction of inflammatory markers, improved cerebral blood flow, and reduction/elimination in vasopressor intervention.

Vitamin C as Antioxidant

Yang's group [117] showed that Vitamin C could provide antioxidant protection to HBOC derived from human cord blood and lowered the MetHb content of HBOC. This led to an important strategy to increase the safety and effectiveness of HBOC. We have confirmed this [17].

Other Approaches

Alayash's group had already reported in detail [5, 56] elsewhere that a Haptoglobin complex could also be used to protect against oxidative stress. Another one is Zal's Arenicola marina Hb [88] with antioxidant activity with more details below. Simon added a pharmacological solution with antioxidant function to their modified Hb.

Preservation for Transplant Organ

A recent promising development included the use HBOCs for the preservation of transplant organ. This would be a less stringent approach since the HBOCs only need to act ex vivo without injection into the body.

Early Basic Research

In 1997, we studied the ex vivo perfusion of isolated small intestine [86] and found that ischemic small intestine released damaging oxygen radicals when reperfused with PolyHb (Fig. 10.4). However, PolyHb-SOD-CAT reperfusion did not increase oxygen radical release. This is important for organ storage for transplantation. Other groups supported this finding in the preservation for rat kidney transplantation by Chang's group from Korea [32] and pancreatic beta cells by Nadithe and Bae [76].

Light's Group

developed a hemoglobin-based oxygen carrier solution (VIR-XV1) for liver allograft preservation in combination with machine perfusion [103]. In order to avoid the complicated use of RBC with Machine Perfusion (MP). they used a bovine-derived glutaraldehyde polymerized hemoglobin-based oxygen carrier (BD HBOC) solution as the perfusate in a swine model with liver allografts preserved with MP (Liver Assist® device) for 12 hours at 21 °C. This success led VirTech Bio to design VIR-XV1, a human derived glutaraldehyde polymerized with a higher molecular weight (MW >500 kD, p50 = 36 mmHg). Histological and EM analysis showed sustained integrity of the hepatic tissue for the first 9 hours. The VIR-XV1 group remained histological and EM intact throughout the entire duration of the experiments while the original BD HBOC group showed progressive endothelial cell damage, early detachment and progressive amount of debris accumulation in the sinusoids from 9 to 12 hours. Both HBOCs provided effective liver oxygenation and CO_2 removal over the 12-hour period.

Zal's Group

prepared a novel natural oxygen carrier with anti-oxidant properties from Arenicola Marina [88]. They used this as an additive to organ preservation solutions for both cold ischemic and reperfusion phases [121]. They reported two studies in IRI animal models (pigs) for kidney and lung transplantation. In kidney transplantation, serum creatinine during first 2 weeks post-transplant improved. Long term follow-up (3 months) confirmed the trend observed in the first 2 weeks. In lung transplantation, 5 hours after there was reduction of graft vascular resistance ($p < 0.05$) and increase in graft oxygenation ratio ($p < 0.05$). Several ischemia-reperfusion related biomarkers showed positive trends in the HEMO2life® group. Thus, HEMO2life® is effective in dealing with the effects of IRI. Clinical trials are ongoing with promising results so far. Cold storage (CS), using an appropriate solution is a standard in liver graft preservation.

However continuous hypothermic machine perfusion (CHMP) is more efficient but more complicated and expensive. They evaluated the use of a CS liver graft solution supplemented with (M101®) on orthotopic pig liver transplantation (OLT). CS + M101 group decreased reperfusion injury compared to CS group. There was no significant difference between CS + M101 and CHMP or CHMP + M101. There was less inflammatory cells activation in CS + M101 group than the CS group.

Nanobiotechnology Based Oxygen Carriers with Enhancement of All Three RBC Functions

Introduction

Sims et al. [98] and Tronstad et al. [101] used a novel microelectrode to measure tissue pCO_2 in animal model of severe hemorrhagic shock. They found that mortality and myocardial ischemia were related to the elevation of tissue pCO_2. Carbonic anhydrase (CA) in red blood cell is the major means for the transport of tissue CO_2 to the lung. The cross-linking of stroma-free hemoglobin to form PolySFHb would contain variable amount of RBC enzyme activity. The enzyme activity of the final product would be vary variable and below that of the RBC.

Polyhemoglobin with Enhanced Catalase-Superoxide Dismutase-Carbonic Anhydrase

We therefore used our nanobiotechnological method to assemble CA with hemoglobin and antioxidant enzymes to form PolyHb-SOD-CAT-CA [10] (Fig. 10.5). It not

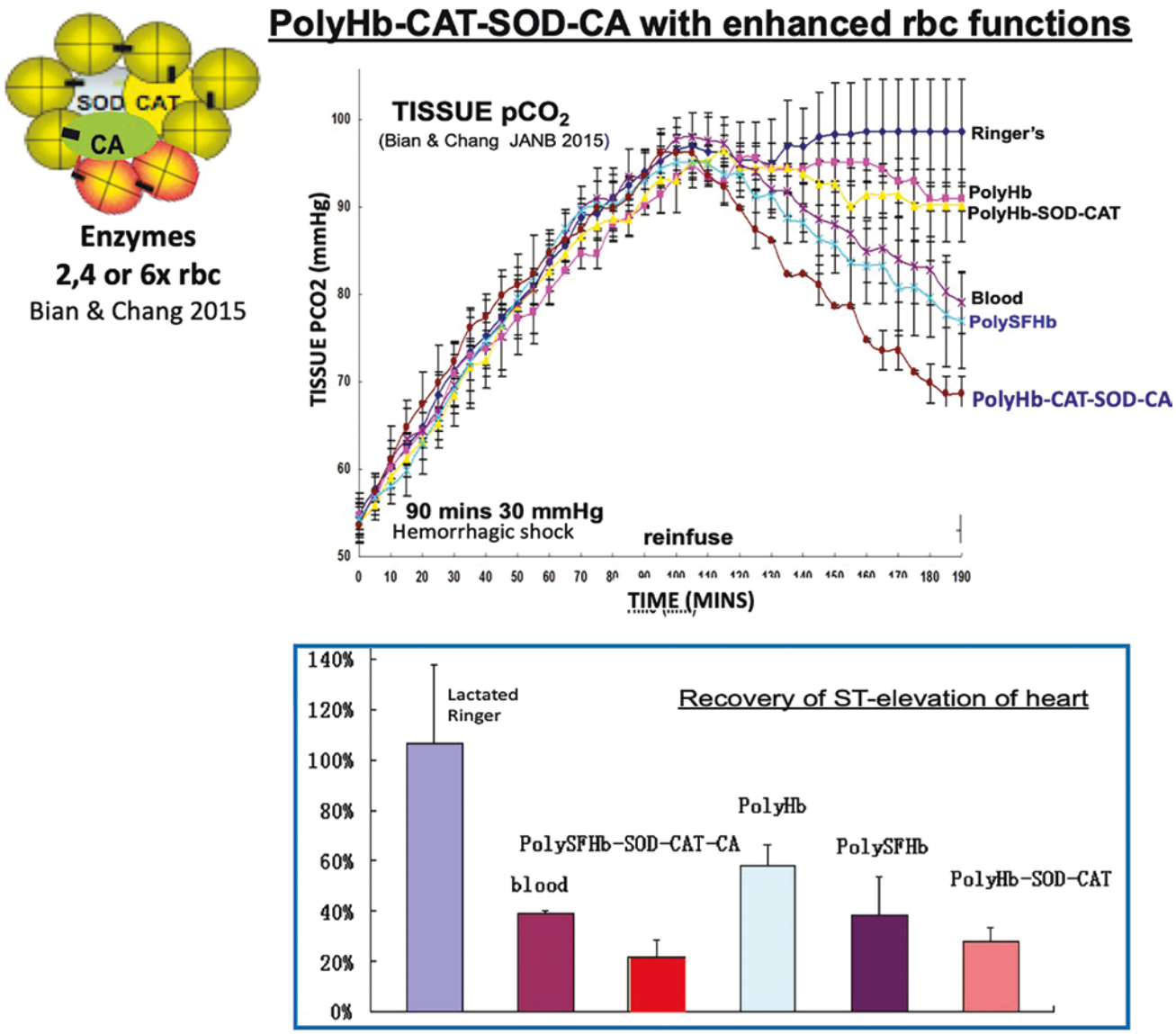


Fig. 10.5 **Upper left:** Polyhemoglobin-catalase-superoxide dismutase-carbonic anhydrase can have up to 6 times red blood cell enzyme concentration. In a rat hemorrhagic shock model with 2/3 blood volume loss and 90 min sustained shock the result is as follow: **Upper right:** significant faster lowering of the elevated tissue pCO_2 and **Lower Right:** faster recovery of the ischemic heart **Middle right:** intestine having better histological finding. **Lower left:** Test for anaphylactic reaction: no significant increase in tryptase nor histamine. Above figures from Chang's group

only had all 3 RBC functions, but it could have enhancement of all 3 RBC functions by increasing the concentrations of RBC enzymes in the complex. These RBC enzymes could be extracted from bovine RBC inexpensively [49]. Our result in a 90 min hemorrhagic shock animal model with 2/3 blood volume loss [10] (Fig. 10.5) showed that it is superior to whole blood in the lowering of elevated intracellular pCO_2, recovery of ST elevation, troponin levels, lowering of elevated lactate, histology of the heart and intestine. It was more efficient than PolyHb and PolySFHb in this sustained 90-minute hemorrhagic shock rat model [10].

We found that PolyHb-SOD-CAT-CA could be lyophilized. Unlike about 1 day for RBC at room temperature, the lyophilized preparation could be stored in room temperature for 320 days [11]. and stable for more than 360 days at 4C as compared to 40 days for donor blood. The lyophilized preparation can be heat pasteurized at 68F for 2 hours. Long term safety study consisted of 4 weekly 5% blood volume infusion followed by 30% volume exchange transfusion [48]**.** The result showed safety and lack of immunological problems. More details are available in a later chapter on this topic.

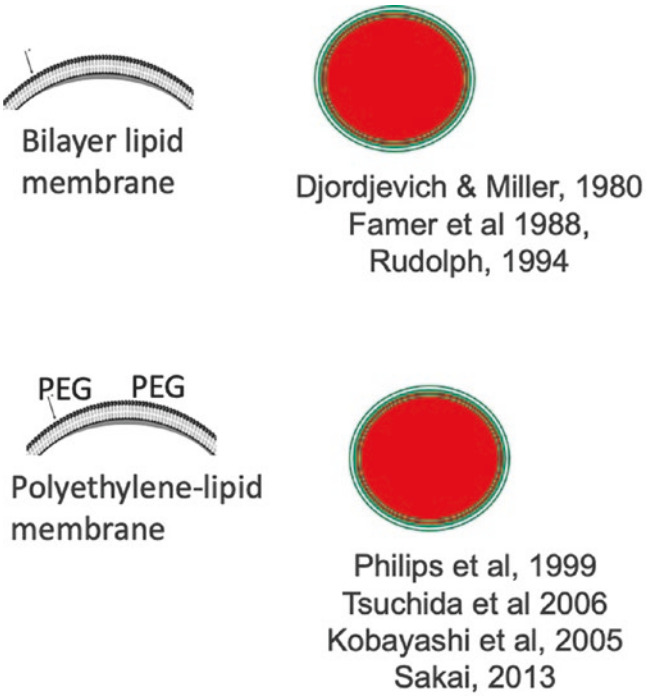


Fig. 10.6 Bilayer lipid membrane and PEG-lipid membrane nano artificial red blood cells

Nanodimension Artificial Red Blood Cells

Early Artificial Red Blood Cells

The first artificial red blood cells prepared by Chang in 1957–1964 (Fig. 10.2) had all the in vitro function of red blood cells as shown by oxygen dissociation curve [18], carbonic anhydrase activity [19] and catalase activities [30]. These artificial red blood cells did not have blood group antigens on the membrane and therefore did no aggregation in the presence of blood group antibodies [22]. However, the single major problem was the rapid removal of these artificial cells from the circulation. Much of the studies since that time were to improve survival in the circulation by decreasing uptake by the reticuloendothelial system. We found that removal of sialic acid from biological red blood cells resulted in their rapid removal from the circulation [20, 22]. This led us to modify the surface properties on artificial red blood cells. This included synthetic polymers, negatively charge polymers, crosslinked protein, lipid-protein, lipid-polymer, addition of surface polysaccharides and others [20, 22].

Bilayer Lipid Membrane Nano Artificial RBC (Fig. 10.6)

In 1965, Bangham [8] reported the preparation of liposomes each consisting of microspheres of onion like concentric lipid bilayers for basic membrane research. The multilamellar liposome limited the amount of water-soluble drugs including hemoglobin that could be enclosed. Thus, they extended Chang's basic principle of preparing artificial cells using ether [18–20] (Fig. 10.2) into what they call an "ether evaporation method" to form single bilayer lipid membrane liposomes for drug delivery [36].

This was extended by Djordjevich & Miller for the preparation of submicron lipid membrane artificial RBC [38] (Fig. 10.6) followed by others including two major groups of Famer, Rudolph et al. in 1988–1994 [43, 89]. Philips et al. in 1999 [82] showed that the circulation half time could be increased to 36 hours in rats by the addition of polyethylene glycol to the lipid membrane. Tsuchida's group [102] started this in Japan followed now by Sakai [90–92]. These advances made it now possible to scale up for detailed preclinical studies leading to the beginning of clinical trials in Japan [92]. More updates and details are available in a later chapter in this book by Sakai.

Nano-Dimension Biodegradable Polymeric Artificial Cells

Using a modification of my 1976 method of micron dimension biodegradable polymeric membrane artificial cells [23] we prepared nano dimension PLA artificial red blood cells [24, 26] and PEG-PLA membrane artificial red blood cells [31].

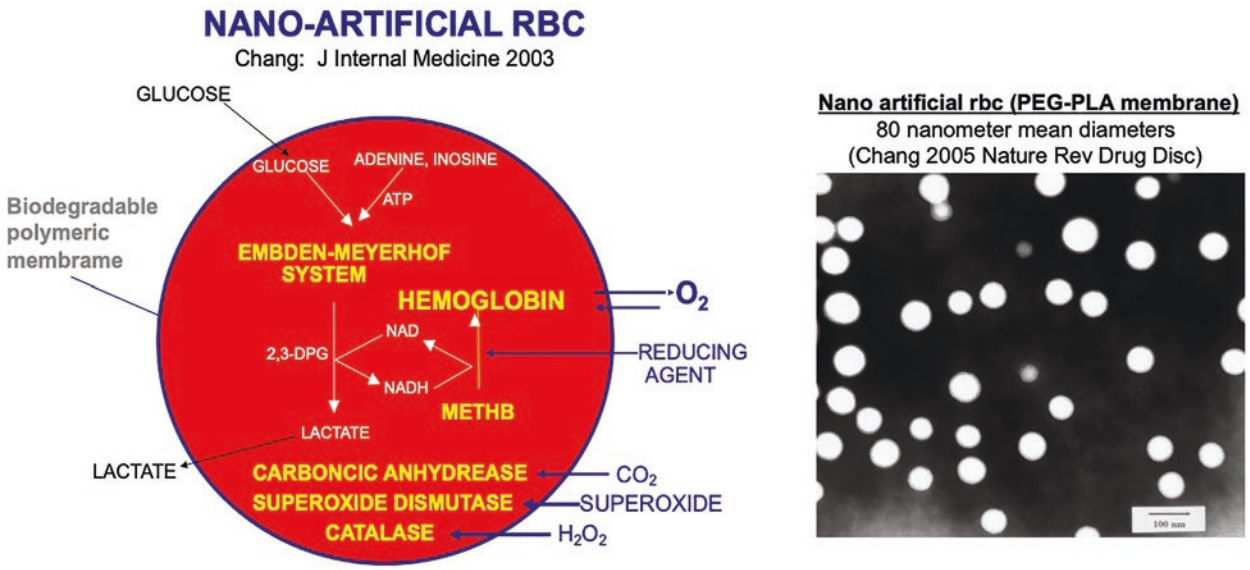


Fig. 10.7 **Right:** E/M photo of nano-dimension PEG/PLA artificial RBC. **Left:** Biodegradable polymeric membrane nano artificial RBC contained hemoglobin and all the enzymes of RBC. The membrane would not be permeable to larger molecules, but freely permeable to glucose and reducing agents from plasma. (With copyright permission from Chang 2007)

Unlike lipid membranes it could be permeable to water soluble small and middle range molecules. Polylactide membrane in PLA nano RBC is biodegradable into lactic acid and finally water and carbon dioxide and thus is not retained in the reticuloendothelial system. These nano artificial RBC of 80–150 nanometers contained all the red blood cell enzymes and can convert methemoglobin to hemoglobin [31] (Fig. 10.7). Addition of a reducing agent, ascorbic acid, prevented the increase of MetHb [31]. Addition of glucose and NADH allowed the Embden Meyerhof enzyme system in the nano artificial RBC to decrease MetHb further [31] (Fig. 10.7).

Using a PEG-polylactide copolymer membrane we were able to increase the circulation time to double that of polyhemoglobin [31]. Infusion of 1/3 blood volume into rats did not have any adverse effects on the kidney [65] or the liver [66] on a long term basis. Our more recent study uses PEG-PLA membrane nano artificial cells containing PolyHb-SOD-CAT-CA in a hemorrhagic shock rat model with 2/3 of the blood removed. After 1 hour of hemorrhagic shock at 30 mmHg, infusion of this preparation effectively resuscitated the animal and lowered the elevated tissue PCO_2 [109].

Variations in the Membrane and Configurations of Nano Artificial RBC

PEG-lipid vesicles were more like lipid–polymer membrane artificial cells [22] and no longer pure lipid vesicles. Thus, polymeric membrane artificial cells have branched off into lipid membrane artificial cells, then PEG-lipid membrane artificial cells, and different types of polymeric membrane artificial cells that are now called by different names including polymersomes, nanocapsules, nanoparticles, vesicles and others. The following are examples of the many possibilities.

Bäumler's group [9] prepared "Hemoglobin-Based Oxygen Carriers HbMP-700" that could deliver more than oxygen. They co-precipitated hemoglobin (Hb) with $MnCO_3$ and immediately followed by the addition of human serum albumin (HSA) then cross-linking. Dissolution of the $MnCO_3$ template resulted in polymerized submicron HbMP-700 with an average size of around 710 ± 60 nm. This did not scavenge NO. The HbMP-700 could be loaded with hydrophobic or hydrophilic nanoparticles (NPs) or with superparamagnetic NPs (e.g. SPIONs) during the precipitation. HbMP-700 were not recognized by phagocytizing cells and they could deliver oxygen to the tissue with low pO_2, could release slowly the immobilized NPs or enzymes and could be detected by MRI if SPIONs were incorporated. Surface modifications with antibodies or peptides would be possible.

Huang's group [107] prepared "Hemosome" by protein–polymer conjugate assembly as oxygen carrier nanoparticle drug delivery system. This was prepared based on the self-aggregated property of proteins in the state of isoelectric point with mild reaction conditions resulting in simple preparation method and good biocompatibility. In this study, albumin and hemoglobin was chosen as model protein for the preparation of empty and drug-loaded nanoparticles. Hemoglobin did not denature or inactivated during the preparation and hemoglobin maintained its ability to transport oxygen.

Komatsu [62] prepared Hemoglobin-Albumin Cluster "HemoAct™" as an Artificial O_2-Carrier**.** They synthesized covalent core-shell structured protein cluster comprising of Hb in the center and human serum albumins (HSA) at the periphery. The covalent linkage was between surface Lys amino groups of Hb and Cys-34 residue of HSA using heterobifunctional cross-linker, α-succinimidyl-ω-maleimido. The HemoAct™ showed higher O_2-affinity (P_{50} = 9 Torr) than the native Hb. Intravenous administration into anesthetized rats did not elicit an unfavorable increase in systemic blood pressure by vasoconstriction. The half-life of [32] I-labeled HemoAct™ in the blood circulation was longer than that of HAS.

These are just a few of the examples since there is no limit to the possibility for variations in configuration [28]. However, as we devise different configurations we should also consider the functional properties.

Nonfunctional or Functional Membrane (Fig. 10.8)

For a given volume of a suspension, the smaller the diameter of the particles, the larger would be the total surface area. Thus, the total surface area of 10 mL nano artificial red blood cells of 100–200 nanometer diameters would be about 100 times that of 10 mL 7 micron (7000 nm) red blood cells. This also means that there is much more total membrane material. Thus, both PEG-lipid and PEG-polylactide nano red blood cells contain substantial amount of nonfunctional lipid or polymeric membrane. On the other hand, for soluble nanobiotherapeutic artificial RBC, PolyHb-SOD-CAT-CA, the "membrane" is functional in the form of oxygen carrying hemoglobin (Fig. 10.4). On the other hand, the membrane enclosed system is more versatile since different materials can be enclosed.

Other Areas

Nanobiotechnology Based Oxygen Carrier with Platelet Function

PolyHb could replace the hemoglobin level in very severe hemorrhage, but in very severe blood loss, platelets also needed to be replaced to avoid inability to clot. We used nanobiotechnology to assemble hemoglobin with fibrinogen to form PolyHb-fibrinogent [115]. We studied this in a rat model and found that replacing more than 80% of the total blood volume with PolyHb leads to defects in blood clotting [115]. Using this, we can replace up to 98% of the total blood volume with PolyHb-fibrinogen without causing clotting problems.

Nanobiotechnology Based Oxygen Carriers with Cancer Suppression Functions

Abnormal microcirculation in tumour leads to decrease in perfusion by oxygen carrying red blood cells. Robinson and Teicher [87] showed that PolyHb can more easily perfuse the abnormal microcirculation of tumours to supply oxygen needed for chemotherapy or radiation therapy Shorr et al. [96] used PEG conjugated hemoglobin for chemotherapy and radiation therapy. Pearce and Gawry [80] showed that PolyHb decreases the growth of tumour and increases the lifespan in a rat model of gliosarcoma brain tumour. Han et al. [50] used PEG-conjugated hemoglobin with cisplatin treatment in a tumor xenograft model, We crosslinked tyrosinase with hemoglobin to form a soluble PolyHb-tyrosinase complex [118]. This has the dual function of supplying the needed oxygen and at the same time lowering the systemic levels of tyrosine needed for the growth of melanoma. Intravenous injections delayed the growth of the melanoma without causing adverse effects in the treated animals. Our

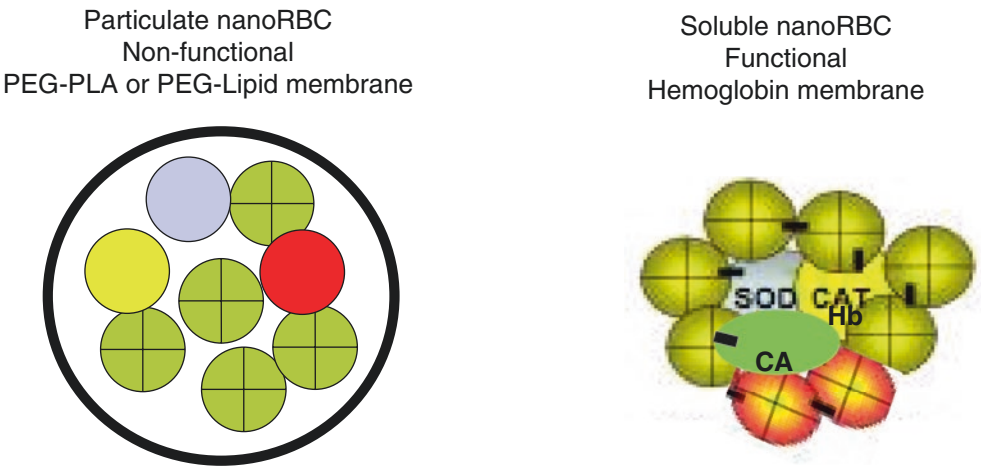


Fig. 10.8 Nonfunctional membrane and functional membrane

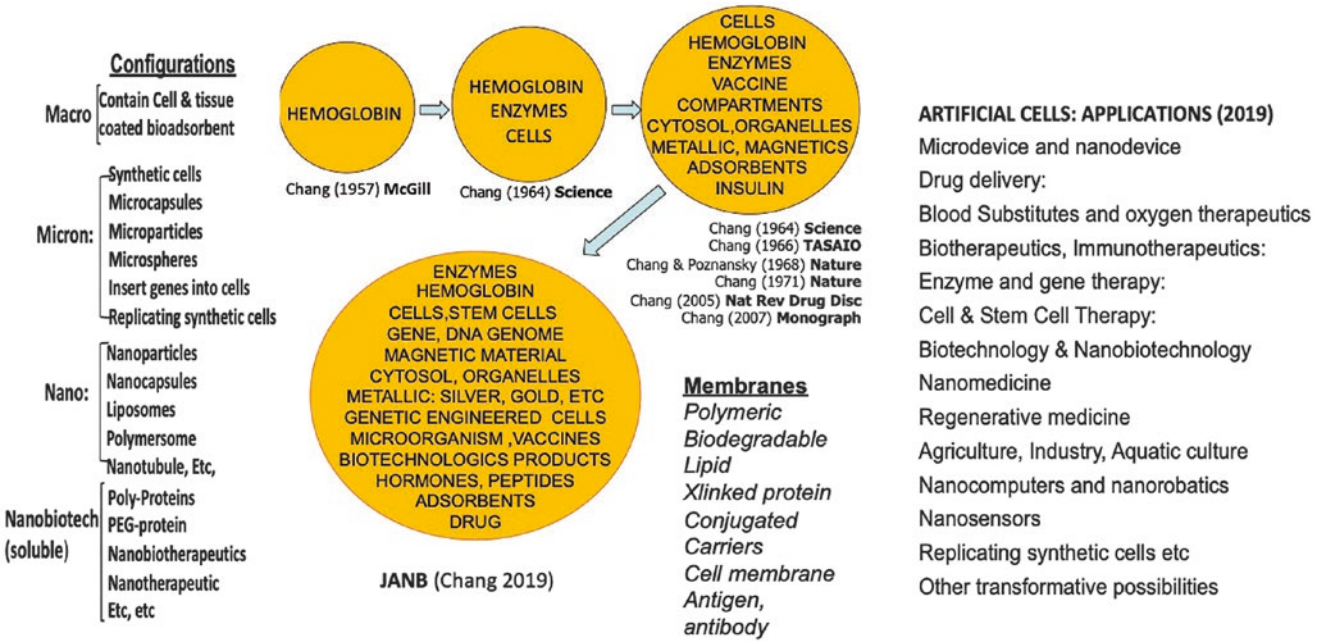


Fig. 10.9 **Middle:** Basic idea of artificial cells that led to different types of early artificial cells. Present status of artificial cells with unlimited variations in contents, membrane material. **Right** variations in dimension, configurations, and terminologies. **Right:** Applications. (From Chang 2019 with copyright permission)

more recent study included the use of PLA membrane nano artificial cells containing polyHb-tyrosinase for intratumour injection in rat melanoma [106] The duo function of oxygen therapeutic and removal of tyrosinae resulted in the suppression of growth of the melanoma [106].

Stem Cells

There is much potential for the use of stem cells for the production different types of blood cells [71]. This may be most useful for platelets and leucocytes since only small amounts are needed. Even then, platelets, unlike nanobiotechnological derived ones, must be stored in room temperature and only for 5 days and have problems related to bacterial contamination. In the case of red blood cells, despite much research, it is still not possible to scale this up sufficiently for the large volume of RBC needed [71] When scale up becomes a reality, this will be an important source of RBC for many clinical conditions.

However, for some uses, these RBC will still have many of the same problems of RBC.

Future Development

There are unlimited possibilities in variations for the artificial cell membranes and contents [28] (Fig. 10.9). Artificial cells can now be of macro, micro, nano and molecular dimensions. Each of these has unlimited variations in configurations. Each configuration resulted in a new terminology that makes the field rather confusing to new comers (Fig. 10.9). This has recently been grouped together [28] (Fig. 10.9). We have only touched the surface of the enormous potential of the extension, innovations and uses of artificial cells (Fig. 10.9). More up to date details are available elsewhere [28].

Summary Discussions

What We Have Learned from Past History

The first nanobiotechnological blood substitutes were reported in the 1960s. Most people thought that blood substitute was a simple matter that could be quickly developed when needed. Thus, blood substitute research was put aside. When H.I.V. came in 1989 there was no blood substitutes, and many patients were infected with H.I.V. contaminated donor blood. It was only then that intense R&D on blood substitutes was belatedly carried out around the world. It was found out too late that blood substitute requires the same long-term research as for any other medical research like cancer and other diseases. Thus, after more than 20 years, only polyhemoglobin was approved for South Africa and Russia.

What Needs to Be Done

Much more research and development are still needed. International progress up to now shows that it is possible to tailor-make blood substitutes ranging from simple to complex. It is urgent to have these ready without again waiting until it is too late. We need to analyze the specific indications for the different generations of blood substitutes. If a condition only needs an oxygen carrier, then there is no need to use a more complex one. On the other hand, it would be folly not to use a more complex one if indicated. We also need to intensify research on the many important ongoing research around the world. These include developing other novel approaches including novel crosslinkers; new sources of material from porcine, bovine, human cord RBC, recombinant, Arenicola marina; basic research on nitric oxide, oxidative stress, haptoglobin, rate of oxygen supply; safety and efficacy analysis and many other areas.

Enormous amounts of resources have been placed into basic research and developments on cancer, rare genetic diseases, molecular biology, and other areas. It is not reasonable to expect that for blood substitutes, we should be expected to come out with a perfect blood substitute with no substantial resources for academic and industrial research and development. Let's not wait for another crisis before we are again forced to do catch-up R & D.

Key Points

- International progress up to now shows that it is possible to tailor-make blood substitutes ranging from simple oxygen carrier to oxygen carrier with enhancement of both antioxidant and carbon dioxide carrier.
- We need to analyze the specific indications for the different types of blood substitutes. If a condition only needs an oxygen carrier, then there is no need to use a more complex one. On the other hand, it would be folly not to use a more complex one if indicated.
- We also need to intensify research on the many important ongoing research around the world These include: develop other novel approaches including novel crosslinkers; new sources of material from porcine, bovine, human cord RBC, recombinant, Arenicola marina; basic research on nitric oxide, oxidative stress, haptoglobin, rate of oxygen supply; safety and efficacy analysis and many other areas.
- Enormous amount of resources has been placed into basic research and developments on cancer, rare genetic diseases, molecular biology, organ failure and other areas. It is not reasonable to expect that for blood substitutes, we should be expected to come out with a perfect blood substitute with little or no resources for academic and industrial research and development.
- Let's not wait for another crisis before we are again forced to do catch-up R & D.

Acknowledgements Material from this chapter comes from some of the text and figures of this author's papers with copy right permission

Material from this chapter comes from some of the text and figures of this author's papers published in Artificial Cells, Nanomedicine and Biotechnology (Taylor & Francis Publisher) with copy right permission

The author's research in this area has been supported by the Canadian Institutes of Health Research and a partnership grant of the Canada Blood Service/Canadian Institutes of Health Research. Both are nonprofit organizations of the government of Canada. The opinions in this paper are those of the author and not necessary those of the granting agencies or the government of Canada.

References

1. Abuchowski A. SANGUINATE® PEGylated bovine hemoglobin with dual carbon monoxide and oxygen delivery agent with anti-inflammatory and anti-vasoconstrictive properties. Abstract 2017 International Symposium on blood substitutes www.artcell.mcgill.ca. 2017.
2. Abuchowski A, Es TV, Palczuk NC, Davis FF. Alteration of immunological properties of bovine serum albumi by convalent attachment of polyethylene glycol. J Biol Chem. 1977;252:2578–3581.
3. Acharya SA, Tsai AG, Intaglietta M. EAF PEG hemoglobins: novel class of non-hypertensive resuscitation fluids: simplicity and advantages of extension arm chemistry for PEGylation. In: Chang TMS, editor. Selected topics in nanomedicine. Singapore: World Science Publisher/Imperial College Press; 2013.
4. Alayash AI. Oxygen therapeutics: can we tame Hb. Nat Rev Drug Discov. 2004;3:152–9.
5. Alayash AI. Unravelling of hemoglobin oxidative toxicity: thirty years of investigation. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
6. Alomari EAH, Ronda L, Bettati S, Mozzarelli A, Bruno S. Modulation of oxygen affinity in hemoglobin-based oxygen carriers. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
7. Amberson WR. Blood substitute. Biol Rev. 1937;12:48.
8. Bangham AD, et al. Diffusion of univalent ions across the lamellae of swollenphospholipids. J Mol Biol. 1965;13:238–52.
9. Bäumler H, Georgieva R. Hemoglobin-based oxygen carriers HbMP-700 can deliver more than oxygen. Biomacromolecules. 2010;11:1480–7.
10. Bian Y, Chang TMS. A novel nanobiotherapeutical Poly-[hemoglobin-superoxide dismutase-catalase-carbonic anhydrase] with no cardiac toxicity for the resuscitation of a 90 minutes sustained severe hemorrhagic shock rat model with 2/3 blood volume loss. Artif Cells Nanomed Biotechnol. 2015;43(1):1–9. http://www.medicine.mcgill.ca/artcell/2015bian&chang.pdf.
11. Bian Y, Guo C, Chang TMS. Temperature stability of Poly-[hemoglobinsuperoxide dismutase–catalase- carbonic anhydrase] in the form of a solution or in the lyophilized form during storage at −80 °C, 4 °C, 25 °C and 37 °C or pasteurization at 70 °C. Artif Cells Nanomed Biotechnol. 2016;44:41–7.
12. Biro G. Rationale, evidence-based transfusion: a physiologist's perspective. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
13. Bucci E. Basic science offers a challenge for developing hemoglobin based oxygen carriers into therapeutic agent. Artif Cells Blood Substit Immobil Biotechnol. 2011;39:206–13.

14. Buehler PW, Haney CR, Gulati A, Ma L, Hsia CJ. Polynitroxyl hemoglobin: a pharmacokinetic study of covalently bound nitroxides to hemoglobin platforms. Free Radic Biol Med. 2004;37(1):124–35.
15. Bunn HF, Jandl JH. The renal handling of hemoglobin. Trans Assoc Am Phys. 1968;81:147.
16. Burhop KE, Estep TE. Hb induced myocardial lesions. Artif Cells Blood Substit Immobil Biotechnol. 2001;29:101–6.
17. Chan G, Chang TMS. Dual effects include antioxidant and prooxidation of ascorbic acid on the redox properties of bovine hemoglobin. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
18. Chang TMS. Hemoglobin corpuscles. Report of a research project for Honours Physiology, Medical Library, McGill University. Also reprinted in full in Chang;s 2007 Monograph on "Artificial Cells". 1957.
19. Chang TMS. Semipermeable microcapsules. Science. 1964;146:524–5.
20. Chang TMS. Semipermeable aqueous microcapsules. Ph.D. Thesis, McGill University. 1965.
21. Chang TMS. Stabilisation of enzymes by microencapsulation with a concentrated protein solution or by microencapsulation followed by cross-linking with glutaraldehyde. Biochem Biophys Res Commun. 1971;44:1531–6.
22. Chang TMS. Artificial cells. Springfield: Charles C. Thomas; 1972. (out of print but available for free online viewing/download at www.artcell.mcgill.ca).
23. Chang TMS. Biodegradable semipermeable microcapsules containing enzymes, hormones, vaccines, and other biologicals. J Bioeng. 1976;1:25–32.
24. Chang TMS. Red blood cell substitutes: principles, methods, products and clinical trials, vol. I (Monograph). Basel: Karger/Landes Systems; 1997. (available for free online viewing at www.artcell.mcgill.ca or www.artificialcell.info).
25. Chang TMS. Therapeutic applications of polymeric artificial cells. Nat Rev Drug Discov. 2005;4:221–35.
26. Chang TMS. Monograph on "ARTIFICIAL CELLS: biotechnology, nanotechnology, blood substitutes, regenerative medicine, bioencapsulation, cell/stem cell therapy". Singapore: World Scientific Publisher/Imperial College Press; 2007, 435 pages. (available for free online viewing/download at www.artcell.mcgill.ca)
27. Chang TMS. Translational feasibility of soluble nanobiotherapeutics with enhanced red blood cell functions. Artif Cells Nanomed Biotechnol. 2017;45:671–6.
28. Chang TMS. ARTIFICIAL CELL evolves into nanomedcine, biotherapeutics, blood substitutes, drug delivery, enzyme/gene therapy, cancer therapy, cell/stem cell therapy, nanoparticles, liposomes, bioencapsulation, replicating synthetic cells, cell encapsulation/scaffold, biosorbent/immunosorbent hemoperfusion/plasmapheresis, regenerative medicine, encapsulated microbe, nanobiotechnology, nanotechnology. Artif Cells Nanomed Biotechnol. 2019;47(1):997–1013. https://doi.org/10.1080/21691401.2019.1577885.
29. Chang TMS. Artificial Cells, Blood Substitutes and Nanomedicine website containing reviews and monographs for free online viewing or download www.artcell.mcgill.ca. 2021.
30. Chang TMS, Poznansky MJ. Semipermeable microcapsules containing catalase for enzyme replacement in acatalsaemic mice. Nature. 1968;218:242–5.
31. Chang TMS, Powanda D, Yu WP. Analysis of polyethyleneglycolpolylactide nano-dimension artificial red blood cells in maintaining systemic hemoglobin levels and prevention of methemoglobin formation. Artif Cells Blood Substit Immobil Biotechnol. 2003;31:231–48.
32. Chang EJ, Lee TH, Mun KC, et al. Effects of polyhemoglobinantioxidant enzyme complex on ischemia-reperfusion in kidney. Transplant Proc. 2004;36:1952–4.
33. Chang TMS, Jiang WH, D'Agnillo F, Razack S. Nanobiotherapeutics as preservation fluids for organs ans cells. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
34. Chang TMS, Bulow L, Jar J, Saika H, Yang CM, editors. Book on Nanobitherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
35. D'Agnillo F, Chang TMS. Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties. Nat Biotechnol. 1998;16:667–71.
36. Deamer DW, Bangham AD. Large-volume liposomes by an ether vaporization method. Biochim Biophys Acta. 1976;443:629–34.
37. DeVenuto F, Zegna A. Blood exchange with pyridoxalated-polymerized hemoglobin. Surg Gynecol Obstet. 1982;155:342.
38. Djordjevich L, Miller IF. Synthetic erythrocytes from lipid encapsulated hemoglobin. Exp Hematol. 1980;8:584.
39. Doherty DH, Doyle MP, Curry SR, et al. Rate of reaction with nitric oxide determine the hypertensive effect of cell free hemoglobin. Nat Biotechnol. 1998;16:672–6.
40. Dudziak R, Bonhard K. The development of hemoglobin preparations for various indications. Anesth. 1980;29:181.
41. Estep TN. Hemoglobin based oxygen carriers, volume expansion, fluid management, and anemia. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
42. Estep TN. Hemoglobin based oxygen carriers and myocardial infarction: assessment of potential mechanisms. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
43. Farmer MC, Rudolph AS, Vandegriff KD, Havre MD, Bayne SA, Johnson SA. Lipsome-encapsulated hemoglobin: oxygen binding properties and respiratory function. Biomater Artif Cells Artif Organs. 1988;16:289–99.
44. Feola M, Gonzalez H, Canizaro PC, Bingham D. Development of bovine stroma-free hemoglobin solution as a blood substitute. Surg Gynecol Obstet. 1983;157:399.
45. Fratantoni JC. Points to consider on efficacy evaluation of hemoglobin and perfluorocarbon based oxygen carriers. Transfusion. 1994;34:712–71.
46. Gould SA, et al. The life-sustaining capacity of human polymerized hemoglobin when red cells might be unavailable. J Am Coll Surg. 2002;195:445–52.
47. Greenburg AG, Kim HW. Evaluating new red cell substitutes: a critical analysis of toxicity models. Biomater Artif Cell Immobil Biotechnol. 1992;20:575–81.
48. Guo C, Chang TMS. Long term safety and immunological effects of a nanobiotherapeutic, bovine poly-[hemoglobin-catalasesuperoxide dismutase-carbonic anhydrase], after four weekly 5% blood volume toploading followed by a challenge of 30% exchange transfusion. Artif Cells Nanomed Biotechnol. 2018;46(7):1349–63. www.artcell.mcgill.ca/safety_immune.pdf.
49. Guo C, Gynn M, Chang TMS. Extraction of superoxide dismutase, catalase and carbonic anhydrase from stroma-free red blood cell hemolysate for the preparation of the nanobiotechnological complex of PolyHemoglobin-superoxide dismutase-catalase-carbonic anhydrase. Artif Cells Nanomed Biotechnol. 2015;43(3):157–62.

50. Han JQ, Yu MH, Dai M, Li HW, Xiu RJ, Liu Q. Decreased expression of MDR1 in PEG-conjugated hemoglobin solution combined cisplatin treatment in a tumor xenograft model. Artif Cells Blood Substit Immobil Biotechnol. 2012;40:239–44.
51. Hoffman SJ, Looker DL, Roehrich JM, et al. Expression of fully functional tetrameric human hemoglobin in Escherichia coli. Proc Natl Acad Sci U S A. 1990;87:8521–5.
52. Hsia JC, Song DL, Er SS, Wong LTL. Pharmacokinetic studies on a raffinose-polymerized human hemoglobin in the rat. Biomater Artif Cell Immobil Biotechnol. 1992;20:587–96.
53. Iwashita Y. Relationship between chemical properties and biological properties of pyridoxalated hemoglobin-polyoxyethylene. Biomater Artif Cell Immobil Biotechnol. 1992;20:299–308.
54. Jahr JS. Hemoglobin-glutamer 250 (bovine) [HBOC-201, Hemopure®] clinical use in South Africa and comprehensive review of cardiac outcomes and risk/benefit in PeerReviewed, indexed studies in humans and animal models. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
55. Jahr JS, Mackenzie C, Pearce LB, Pitman A, Greenburg AG. HBOC-201 as an alternative to blood transfusion: efficacy and safety evaluation in a multicenter phase III trial in elective orthopaedic surgery. J Trauma. 2008;64:1484–97.
56. Jia YP, Alayash AI. Molecular basis of haptoglobin and hemoglobin complex formation and protection against oxidative stress and damage. In: Chang TMS, editor. Selected topics in nanomedicine. Singapore: World Science Publisher/Imperial College Press; 2013.
57. Keipert P, Chang TMS. In-vivo effects of total and partial isovolemic exchange transfusion in fully conscious rats using pyridoxalated polyhemoglobin solution as a colloidal oxygen-delivery blood substitute. Vox Sang. 1987;53:7–14.
58. Kettisen K, Bülow L. Cysteine mutations in recombinant fetal hemoglobin influence oxidative side-reactions. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
59. Kim HW, Greenburg AG, editors. Hemoglobin-based oxygen carriers as red cell substitutes and oxygen therapeutics. New York, London: Springer; 2014. 738 pages.
60. Klein HG. The prospects for red-cell substitutes. N Engl J Med. 2000;342(22):1666–8.
61. Kobayashi K, Tsuchida E, Horinouchi H, editors. Artificial oxygen carriers. Singapore: Spring; 2005.
62. Komatsu T. Hemoglobin-albumin cluster "HemoAct™" as an artificial O_2-carrier. Abstract, 2017 International Symposium on Blood Substitutes. www.artcell.mcgill.ca. 2017.
63. Li XZ, Zhang XW, Liu Q. Determination of the molecular weight distribution of PEGylated bovine hemoglobin (PEG-bHb). Artif Cells Blood Substit Immobil Biotechnol. 2005;33:13–28.
64. Li T, Yu R, Zhang HH, Liang WG, Yang XM, Yang CM. A method for purification and viral inactivation of human placenta hemoglobin. Artif Cells Blood Substit Immobil Biotechnol. 2006;34(2):175–88.
65. Liu ZC, Chang TMS. Effects of PEG-PLA-nano artificial cells containing hemoglobin on kidney function and renal histology in rats. Artif Cells Blood Substit Immobil Biotechnol. 2008;36:421–30.
66. Liu ZC, Chang TMS. Long term effects on the histology and function of livers and spleens in rats after 33% toploading of PEG-PLA-nano artificial red blood cells. Artif Cells Blood Substit Immobil Biotechnol. 2008;36:513–24.
67. Liu Q, Xiu RJ. Proceedings of the XI ISBS Symposium at Peking Union Medical College, Chinese Academy of Medical Sciences. Artif Cells Blood Substit Immobil Biotechnol. 2008;36(3):169–293.
68. Liu XB, Li CX, Qi X, Wong BL, Man K. The application of hemoglobin-based oxygen carriers in liver cancer treatment in rodent models. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Wahington D.C.: World Science Publisher; 2021.
69. Looker D, Abbott-Brown D, Cozart P, et al. A human recombinant hemoglobin designed for use as a blood substitute. Nature. 1992;356:258–60.
70. Ma L, Hsia CJC. Polynitroxylated hemoglobin as a multifunctional therapeutic for critical care and transfusion medicine. In: Chang TMS, editor. Selected topics in nanomedicine. Singapore: World Science Publisher/Imperial College Press; 2013.
71. Mazurier C, Douay L, Lapillonne H. Red blood cells from induced pluripotent stem cells: hurdles and developments. Curr Opin Hematol. 2011;18:249–53.
72. Mer M, Hodgson E, Wallis L, Jacobson B, Levien L, Snyman J, Sussman MJ, James M, van Gelder A, Allgaier R, Jahr JS. Hemoglobin glutamer-250 (bovine) in South Africa: consensus usage guidelines from clinician experts who have treated patients. Transfusion. 2016;56:2631–16.
73. Moore E, Moore FA, Fabian TC, Bernard AC, Fulda GJ, Hoyt DB, et al. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. J Am Coll Surg. 2009;208:1–13.
74. Moss GS, Gould SA, Sehgal LR, Sehgal HL, Rosen AL. Hemoglobin solution - from tetramer to polymer. Biomater Artif Cells Artif Organs. 1988;16:57–69.
75. Nadithe V, Bae YH. Hemoglobin conjugates with antioxidant enzymes (hemoglobin-superoxide dismutase-catalase) via poly(ethylene glycol) crosslinker for protection of pancreatic beta RINm5F cells in hypoxia. Tissue Eng. 2011;17:24532462.
76. Nadithe V, Bae YH. Hemoglobin conjugates with antioxidant enzymes (hemoglobin-superoxide dismutase-catalase) via poly(ethylene glycol) crosslinker for protection of pancreatic beta RINm5F cells in hypoxia. Tissue Eng. 2011;17:2453–62.
77. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA. 2008;299:2304–12.
78. Ono-Uruga Y, Tozawa K, Ikeda Y, Matsubara Y. Megakaryocytes and platelets from novel human adipose tissue-derived mesenchymal stem cells: development of cell based regenerative medicine. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
79. Park YK, Abuchowski A, Davis S, Davis F. Pharmacology of Escherichia coli-L-asparaginase polyethylene glycol adduct. Anticancer Res. 1981;1:373–6.
80. Pearce LB, Gawryl MS. Overview of preclinical and clinical efficacy of Biopure's HBOCs. In: Chang TMS, editor. Blood substitutes: principles, methods, products and clinical trials, vol. 2. Basel: Karger; 1998. p. 82–98.
81. Perutz MF. Stereochemical mechanism of oxygen transport by hemoglobin. Proc R Soc Lond B. 1980;208:135.
82. Philips WT, Klpper RW, Awasthi VD, Rudolph AS, Cliff R, Kwasiborski VV, Goins BA. Polyethylene glyco-modified liposome-encapsulated Hb: a long circulating red cell substitute. J Pharmacol Exp Ther. 1999;288:665–70.
83. Powanda D, Chang TMS. Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. Artif Cells Blood Substit Immobil Biotechnol. 2002;30:25–42.
84. Przybelski R, Blue J, Nanvaty M, Goldberg C, Estep T, Schmitz T. Clinical studies with diaspirin cross-linked hemoglobin solution (DCLHb™): a review and update. Artif Cells Blood Substit Immobil Biotechnol. 1996;24(abstracts issue):407.

85. Rabiner SF, Helbert JR, Lopas H, Friedman LH. Evaluation of stroma free hemoglobin solution as a plasma expander. J Exp Med. 1967;126:1127.
86. Razack S, D'Agnillo F, Chang TMS. Crosslinked hemoglobin-superoxide dismutase-catalase scavenges free radicals in a rat model of intestinal ischemia reperfusion injury. Artif Cells Blood Substit Immobil Biotechnol. 1997;25:181–92.
87. Robinson MF, Dupuis NP, Kusumoto T, Liu F, Menon K, Teicher BA. Increased tumor oxygenation and radiation sensitivity in two rat tumors by a hemoglobin-based oxygen carrying preparation. Artif Cells Blood Substit Immobil Biotechnol. 1995;23:431–8.
88. Rousselot M, Delpy E, Drieu La Rochelle C, Lagente V, Pirow R, Rees JF, Hagege A, Le Guen D, Hourdez S, Zal F. Arenicola marina extracellular hemoglobin: a new promising blood substitute. Biotechnol J. 2006;1(3):333–45.
89. Rudolph AS. Encapsulated hemolgobin: current issues and future goals. Artif Cells Blood Substit Immobil Biotechnol. 1994;22:347–60.
90. Sakai H. Biocompatibility of a highly concentrated fluid of hemoglobin-vesicles as a transfusion alternative. In: Chang TMS, editor. Selected topics in nanomedicine. Singapore: World Science Publisher/Imperial College Press; 2013.
91. Sakai H, Yamada M. Prevention of methemoglobin formation in artificial red cells (hemoglobin-vesicles). In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
92. Sakai H, Azuma H, Horinouchi H, Kobayashi K. Translational research of Hbvesicles (artificial red cells) for a transfusion alternative and O_2/CO therapeutics. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
93. Savitsky JP, Doozi J, Black J, Arnold JD. A clinical safety trial of stroma free hemoglobin. Clin Pharmacol Ther. 1978;23:73.
94. Sheng Y, Yuan Y, Liu C, Tao X, Shan X, Xu F. In vitro macrophage uptake and in vivo biodistribution of PLA–PEG nanoparticles loaded with hemoglobin as blood substitutes: effect of PEG content. J Mater Sci Mater Med. 2009;20(9):1881–91.
95. Shoemaker S, Gerber M, Evans G, Paik L, Scoggin C. (Somatogen Co.USA). Initial clinical experience with a rationally designed genetically engineered recombinant human hemoglobin. Artif Cells Blood Substit Immobil Biotechnol. 1994;22:457–65.
96. Shorr RG, Viau AT, Abuchowski A. Phase 1B safety evaluation of PEG hemoglobin as an adjuvant to radiation therapy in human cancer patients. Artif Cells Blood Substit Immobil Biotechnol. 1996;24(abstracts issue):407.
97. Simoni J, Simoni G, Lox CD, Prien SD, Shires GT. Modified hemoglobin solution with desired pharmacological properties does not activate nuclear transcription factor NF-kappa B in human vascular endothelial cells. Artif Cells Blood Substit Immobil Biotechnol. 1997;25:193–210.
98. Sims C, Seigne P, Menconi M, Monarca J, Barlow C, Pettit J, Puyana JC. Skeletal muscle acidosis correlates with the severity of blood volume loss during shock and resuscitation. J Trauma. 2001;51:1137–46.
99. Tam SC, Blumenstein J, Wong JT. Dextran hemoglobin. Proc Natl Acad Sci USA. 1976;73:2128.
100. Timm B, Elmer J. Enhancing the stability of Lumbricus terrestris Erythrocruorin (LtEc) for use as a blood substitute. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
101. Tronstad C, Pischke SE, Holhjem L, Tonnessen TI, Martinsen OG, Grimnes S. Early detection of cardiac ischemia using a conductometric PCO 2 sensor: real-time drift correction and parameterization. Physiol Meas. 2010;31:1241–55.
102. Tsuchida E, Sakai H, Horinouchi H, Kobayashi K. Hemoglobin Vesicles. Artif Cell Blood Substit Biotechnol. 2006;34:581–8.
103. Vandegriff KD, Malavalli A, Light WR. Development of a new HBOC for liver allograft preservation in combination with machine perfusion−clearing the wait list. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
104. Vincent JL, Privalle CT, Singer M, Lorente JA, DeAngelo J. Multicenter randomized, placebo-controlled phase III study of pyridoxalated hemoglobin polyoxyethylene in distributive shock (PHOENIX)*. Crit Care Med. 2015;43(1):57–64.
105. Walder JA, Zaugg RH, Walder RY, Steele JM, Klotz IM, IM. Diaspirins that cross-link alpha chains of hemoglobin: bis(3,5-dibromosalicyl) succinate and bis(3,5-dibormosalicyl) fumarate. Biochemistry. 1979;18:4265–70.
106. Wang Y, Chang TMS. A polymer-lipid membrane artificial cell nanocarrier containing enzyme-oxygen biotherapeutic inhibits the growth of B16F10 melanoma in 3D culture and in a mouse model. Artif Cells Nanomed Biotechnol. 2021;49(1):461–70.
107. Wang Y, Huang Y. "Hemosome" by protein−polymer conjugate assembly as oxygen carrier. Abstract. 2017 International Symposium on Blood Substitutes. www.artcell.mcgill.ca. 2017.
108. Wang Y, et al. Polymerized human placenta hemoglobin (PolyPHb) attenuates myocardial infarction injury in rats. Artif Cells Blood Substit Immobil Biotechnol. 2012;40(1–2):7–13.
109. Wei G, Bian YZ, Chang TMS. PLA-PEG nanoencapsulated nano artificial red blood cells that act as O_2 and CO_2 carrier with enhanced antioxidant activity: polyhemoglobin-superoxide dismutase-catalase-carbonic anhydrase. Artif Cells Nanomed Biotechnol. 2013;41(4):232–9.
110. Weiskopf RB. Hemoglobin-based oxygen carriers: disclosed history and the way ahead: the relativity of safety. Anesth Analg. 2014;119:758–60.
111. Wetzler M, Sanford BL, Kurtzberg J, DeOliveira D, Frankel SR, Powell BL, Kolitz JE, Bloomfield CD, Larson RA. Effective asparagine depletion with pegylated asparaginase results in improved outcomes in adult acute lymphoblastic leukemia: cancer and leukemia group B study 9511. Blood. 2007;109(10):4164–7.
112. Winslow RM, editor. Blood substitutes. Amsterdam: Academic; 2006.
113. Wollocko H, Harrington J, Jahr JS, Steier K, Wollocko J. OxyVita® Hb: a step forward in delivering oxygen carrying capacity for therapeutic applications. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. World Science Publisher; 2021.
114. Wong JT. Rightshifted dextran-hemoglobin as blood substitute. Biomater Artif Cells Artif Organs. 1988;16:237–45.
115. Wong N, Chang TMS. Polyhemoglobin-fibrinogen: a novel blood substitutes with platelet-like activity for extreme hemodilution. Artif Cells Blood Substit Immobil Biotechnol. 2007;35:481–9.
116. Wong BL, Kwok SY. Method for the preparation of a heat stable oxygen carriercontaining pharmaceutical composition, United States Patent 7,932,356, April 26, 2011.
117. Yang CM, Liu J, Zhao TM, editors. Chinese transfusion medicine. Beijing: People's Medical Publishing House; 2021, 937 pages
118. Yu BL, Chang TMS. In vitro and in vivo effects of polyhemoglobintyrosinase on murine B16F10 melanoma. Melanoma Res J. 2004;14:197–202.
119. Yu B, Zapol WM. Hemoglobin-based oxygen carriers and inhaled nitric oxide. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
120. Yu BL, Shahid M, Egorina EM, Sovershaev MA, Raher MJ, Lei C, Wu MX, Bloch KD, Zapol WM. Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric

oxide by a hemoglobin-based oxygen carrier. Anesthesiology. 2010;112:586–94.
121. Zal F. Use of HEMO2life - an innovative oxygen carrier in organ transplantation. Abstract, 2017 International Symposium on Blood Substitutes. www.artcell.mcgill.ca. 2017.
122. Zhoa L. Strategies to decrease the oxidative toxicity of hemoglobin-based oxygen carriers. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
123. Zhu YJW, Chen C. Glutaraldehyde polymerized porcine haemoglobin. In: Chang TMS, editor. Selected topics in nanomedicine. Singapore: World Science Publisher/Imperial College Press; 2013.
124. Zhu H, Yan K, Dang X, Huang H, Chen E, Chen B, Chao L, Chang TMS, Dai P, Chen C. Immune safety evaluation of polymerized porcine hemoglobin (pPolyHb) - a potential red blood cell substitutes. In: Chang TMS, Bulow L, Jahr JS, Sakai H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
125. Zuck TF. Difficulties in demonstrating efficacy of blood substitutes. Artif Cells Blood Substit Immobil Biotechnol. (Guest editor: R.Winslow). 1994;22:945–54.

Part II

Pharmacology and Physiology of Oxygen Therapeutics

Classifications of Blood Substitutes

11

Henry Liu, Alan D. Kaye, Thomas Verbeek,
Kristin Brennan, Rageev Dalal, Patrick McQuillan,
and Jonathan S. Jahr

Introduction

Blood is a vital substance for human life. The need for blood replacement may be traced as far back as human history. The ancient medical practitioners tried numerous substances, such as milk, plant resins, beer, sheep blood, urine, as a replacement for human blood [1, 2]. Among many other functions, the most important function of blood is to carry oxygen from the lungs to all organs and tissues of the body [3, 4]. The ancient purpose of blood replacement was not all for its life-saving oxygen-carrying function, but believed that replacement of a person's blood might be able to cure some diseases or improving a personality [2]. In modern times, there are great demands worldwide for allogeneic blood transfusions for various life-saving clinical scenarios [4, 5]. However, the human blood for transfusion has almost always been in short supply [5], therefore, there exists an enormous need for blood substitute products. Since the key function of blood is transporting oxygen to the tissues to meet the demand for oxygen in metabolic activities, and the most sought-after purpose is the oxygen-carrying function of hemoglobin, erythrocyte substitute treatment is thus considered as oxygen therapeutics [6]. Without any doubt, allogeneic blood shortage is not the only driving force for blood substitutes, complications and side effects related to allogeneic blood transfusion as well as some patients' refusal to human blood due to religious reasons, are also major forces behind the enthusiastic pursuit of alternatives to human blood. Since 1970's, significant efforts have been made to develop blood substitutes for many clinical indications. The potential clinical benefits of blood substitutes include universal compatibility, prolonged shelf-life, storage at room temperature, no disease transmissions, no antigenic reactions, no immunologic effects, enhanced oxygen delivery, potential abundant supply, and improved rheologic properties. There are numerous products in different stages of development worldwide. Some products achieved governmental approval for the use in human patients, some obtained governmental approval but later being un-shelved due to various clinical problems encountered during clinical trials or use in human patients, some are undergoing various phases of clinical trials, and some are still in laboratory experimental stages. Due to the complexities of the mechanisms and ever-increasing applications of technologies for the development of blood substitutes-related products, concise and practical classifications seem to be imperative for health care providers have a better understanding of this venue of interventions in patients who are in dread need of improving their oxygen transportation and saving their lives or avoiding severe side effects of allogeneic blood transfusion [7].

H. Liu (✉)
Anesthesiology & Critical Care, University of Pennsylvania, Philadelphia, PA, USA

A. D. Kaye
Department of Anesthesiology and Department of Pharmacology, Toxicology, and Neurosciences, LSU School of Medicine, Shreveport, LA, USA

Department of Anesthesiology and Pharmacology, LSU School of Medicine, New Orleans, LA, USA

Department of Anesthesiology and Pharmacology, Tulane School of Medicine, New Orleans, LA, USA

T. Verbeek · K. Brennan · R. Dalal · P. McQuillan
Department of Anesthesiology and Perioperative Medicine, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, PA, USA

J. S. Jahr
Department of Anesthesiology and Perioperative Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Blood Composition

Human blood is composed of liquid plasma (about 55%) and formed elements (roughly 45%). Plasma appears light-yellowish or straw-colored grossly and functions as the liquid base for whole blood, and plasma contains various amount of water (91%), proteins (7%, albumin, globulin, fibrinogen),

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_11

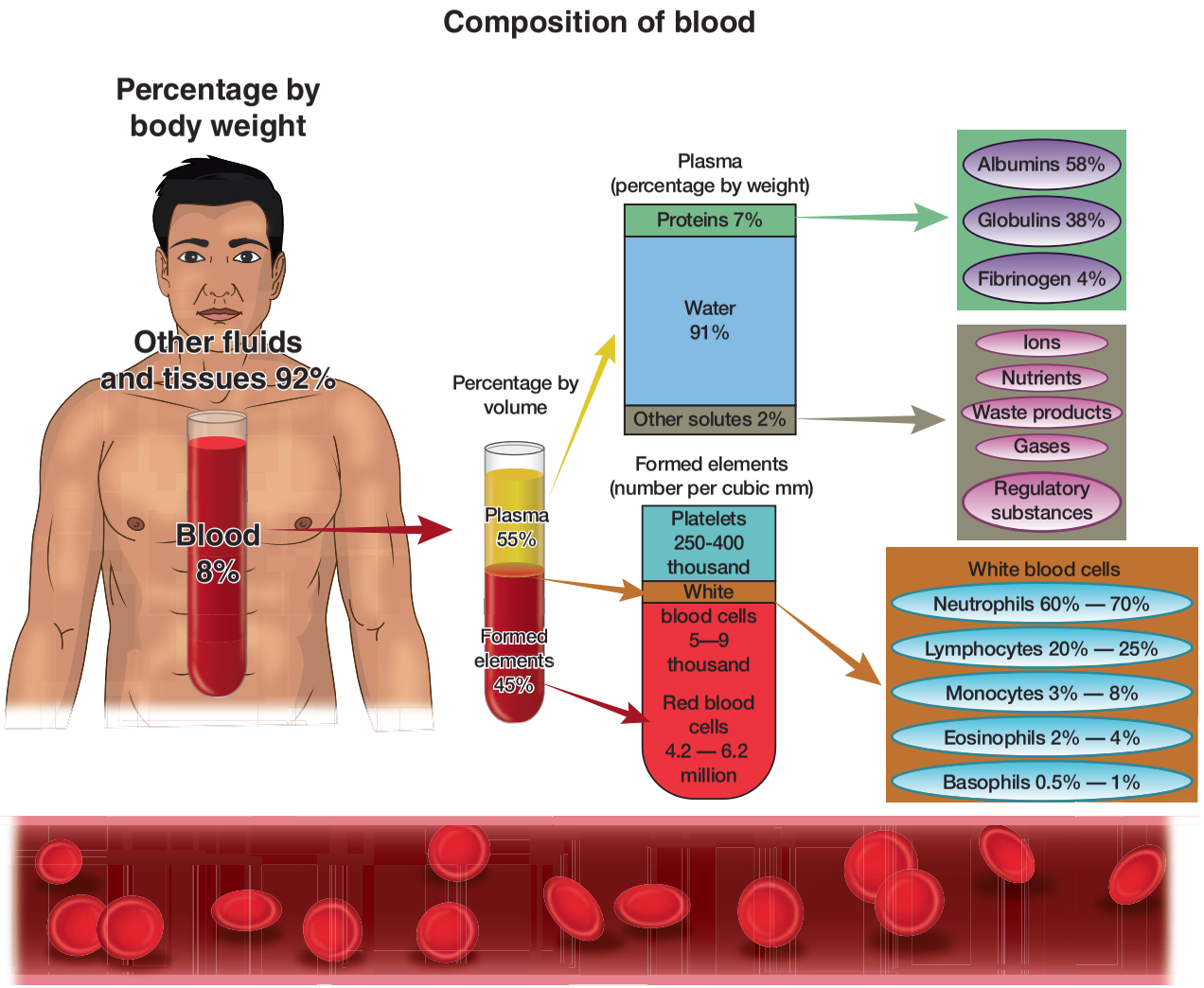


Fig. 11.1 Human blood components

and other solutes (nutrients, ions, waste products, gases, regulatory substances). Formed elements include red blood cells (erythrocyte, RBC), white blood cells (leukocyte, WBC) and platelets (thrombocyte) [3, 4], as illustrated in Fig. 11.1. Thus, classifications of blood substitutes will include substitutes for RBC, WBC, platelet, and plasma. Blood component therapy will be discussed in separate chapter in this book.

Classification of Blood Substitutes

Classification Based on the Blood Components

Red Blood Cell Substitutes

RBC substitutes, produced to carry oxygen from the lungs to the organs and tissues of the body as alternatives to RBC, currently include the following four major categories: hemoglobin-based oxygen carriers (HBOCs, also interchangeably termed "Modified hemoglobin solutions" in some literature), perfluorocarbon-based oxygen carriers (PFBOCs), genetically engineered recombinant hemoglobin, artificial and cultured erythrocytes. RBC substitutes are basically prepared for the replacement of erythrocyte's oxygen-carrying function [6, 7]. This group can be further classified as described later in this chapter.

White Blood Cell (Leukocyte) Substitutes

White blood cells (WBC, leukocyte) are the basic component of the human immune system involved in protecting our body against foreign invaders and infectious pathogens. All WBCs are produced from hematopoietic stem cells in the bone marrow. WBCs are present throughout the whole human body. In patients with severe leukocytopenia, granulocyte transfusions have been proved to be an effective therapeutic intervention for progressive infections. However, granulocyte transfusion has not been widely accepted because it has been extremely difficult to transfuse adequate quantity of compatible granulocytes. As recombinant granulocyte colony-stimulating factor is increasingly available and used, it is probable to produce large number of granulocytes by stimulating normal donors [8].

Platelet Substitutes

Platelets are one of the cellular components in the blood. Platelets facilitates hemostasis by a cascade of reactions as platelet adhesion, activation and aggregation. Fibrinogen is an essential protein in thrombus formation and platelet aggregation. So many so-called platelet substitutes contain fibrinogen molecules as well. Platelet substitutes can be cellular as thromboerythrocytes, or non-cellular as liposomal derivatives or fibrinogen-coated albumin particles [9]. Platelet substitutes can be classified as following:

(a) **Cellular:**
1. Modified Erythrocytes or Thromboerythrocytes by binding peptides containing the RGD (Arg-Gly-Asp) sequence to erythrocytes. These erythrocytes will selectively bind to activated platelets. The erythrocytes have no significant change in their rheological properties [9].
2. Modified Erythrocytes or Thromboerythrocytes by adding peptides with the H12, the fibrinogen γ-chain dodecapeptide.
3. Plateletsomes: liposomes containing various amount of platelet membrane proteins as GPIIb-3, GPIb, and GPVI/III [10]

(b) **Acellular hemostatic agents**
1. Fibrinogen-coated albumin particles: Thrombospheres, Synthocytes
2. Agents to improve platelet function: antifibrinolytic agents, recombinant activated Factor VII
3. Thrombopoietic agents: Interleukin 11 (IL-11)

Plasma Substitutes

Administration of various blood plasma substitutes is probably one of the most common medical interventions in clinical practice. Plasma replacement is also considered a blood volume replacement. Though no ideal plasma substitute exists, many commercially available plasma substitute products are being used on daily basis in medical facilities worldwide [11, 12]. These products can be classified as crystalloid solutions, natural colloid solution and synthetic colloid products.

(a) **Crystalloid solution**
1. Normal saline: Normal saline contains sodium 154 mmol/L and Chloride 154 mmol/L with 278 mOsmol/L and a pH of 5.5. Normal saline does not have potassium, so it is good for patients with hyperkalemia such as renal patients.
2. Lactated Ringer's solution: Lactated Ringers contain Sodium 130 mmol/L, Chloride 109 mmol/L, Potassium 4 mmol/L, and Lactate 28 mmol/L. Lactated Ringer's solution is hypotonic with 276 mOsm/L and a pH of 6.5.
3. Plasmalyte A and congeners: Plasmalyte A contains Sodium 140 mmol/L, Chloride 98 mmol/L, Potassium 5 mmol/L, Magnesium 3 mmol/L, Acetate 27 mmol/L and Gluconate 23 mmol/L with 294 mOsm/L and a pH of 7.4.

(b) **Natural colloid**: Albumin is a naturally existing protein in human plasma.

(c) **Synthetic colloid**
1. Hydroxyethyl Starches: Hydrolyzed derivatives of amylopectin from cornstarch.

2. Dextrans: A complex branched polymerized polysaccharides derived from the condensation of glucose.
3. Gelatins: A group of products formed from collagen hydrolysis.

Classification of Erythrocyte Substitutes

Hemoglobin-Based Oxygen Carriers

HBOC was first attempted in 1933 by Dr. Amberson who showed that bovine hemolysates could transport oxygen in mammals [13]. Then in 1943, Dr. Amberson again pioneered in experimenting acellular hemoglobin solution as an alternative to human blood [14]. These initially studied cell-free hemoglobin solutions were not a viable replacement of allogeneic human blood because they caused many side effects, often times fatal [15]. The most common side effects were hypertension, acute renal failure and cardiac toxicities [1, 14]. The first study on microencapsulated hemoglobin was reported in 1957 [16]. After over two decades of dormant period, the search for blood substitutes was re-ignited by the discovery of HIV and boosted by the blood transfusion-related disease transmissions (AIDS, hepatitis) in the 1980s. Stable, safe, and most importantly affordable alternative products to human blood will lead to higher availability of oxygen-carrying products to the world population, and hence reduce global inequality of blood product supply, which has been a focus point of the World Health Organization (WHO) for the millennium. WHO believes synthetic biology and metabolic engineering technology developments have created a unique opportunity to construct promising candidates for hemoglobin production [17–19]. The diverse and complex challenges are no longer insurmountable with the recent development in powerful technologies provided by synthetic biomedical and genetic engineering technologies. HBOCs now become the most common category of alternatives to allogeneic human blood. It is also the most investigated and has the most prototypes or developed products [6].

Based on the mechanisms of hemoglobin molecules being biochemically modified, protectively packaged or produced, HBOCs can be categorized as following (Table 11.1), (Fig. 11.2):

(a) **Cross-linked hemoglobin tetramer**: The cross-linked tetramer stabilizes the molecule and can prevent renal filtration [6, 18]. The intermolecular cross-linking between the two α- and the two β- subunits using a site-specific crosslinker, as HemAssist (diaspirin cross-linked hemoglobin, Baxter, failed Phase III clinical trial).

(b) **Polymerized hemoglobin tetramers**: the surface amino acid groups are linked by reagents like glutaraldehyde or O-raffinose. Polymerization of hemoglobin molecules can attenuate some intrinsic problems with tetrameric hemoglobin infusion: short intravascular retention, reduced colloid osmotic activity, and vasoconstriction. Hemoglobin tetramer with MW 65kD or less can penetrate through the vascular endothelial layer to bind and exhaust abluminal nitric oxide (NO), leading to severe vasoconstriction, while polymerized hemoglobin with more than 130KD stays intravascularly [20], only binding luminal NO. Some minor side effects have been reported with this compound. These polymerized hemoglobin molecules are produced with one of the following linkers:
   - Glutaraldehyde: HemoPure (from bovine Hb) and PolyHeme (from human Hb)
   - O-Raffinose: OxyVita (a bovine polyhemoglobins) can be stored at room temperature for more than 1 year. Hemolink is also cross-linked with O-Raffinose.

(c) **Conjugated tetramers**: By attaching large molecules to the surface groups of hemoglobin, the hemoglobin molecules can be "enlarged", thus significantly decreased their extravasation and have significantly longer half-life in the blood circulation [6]. Yet these hemoglobin molecules are still much smaller than RBCs and can be more accessible to much smaller capillary networks. Therefore, they are very favorable for stroke patients, and can be also used to increase tumor sensitivity to chemotherapy. Three large molecules have been used for this hemoglobin-conjugation to create larger hemoglobin molecules:
   - Maleimide-PEGylated human hemoglobin: Hemospan by Sangart, Inc.
   - PEGylated bovine carboxy- hemoglobin: Sanguinate by Prolong Pharmaceuticals
   - Pyridoxalated Hemoglobin polyoxyethylene (PHP) conjugate: Hemoximer by Apex Bioscience (Phase III terminated in 2011 in Europe)

(d) **Cross-linked and polymerized**: PolyHeme/SFH-P, cross-linking stroma-free human hemoglobin, polymerized with glutaraldehyde, by Northfield inc [21]

(e) **Cross-linked and conjugated**: PHP (Hemoximer) by Apex Bioscience can also be categorized to this category.

(f) **Natural acellular hemoglobin**: A natural extracellular hemoglobin isolated from the polychaete Arenicola marina (Hemarina, by Hemarina SA, France) is hopeful for future clinical applications [22]. Another natural hemoglobin molecule of the common earthworm, Lumbricus terrestris, is a polymer with a molecular weight of 400 KD that can carry oxygen and circulate intravascularly [23].

(g) **Liposome-encapsulated hemoglobin**: the hemoglobin molecules are basically encapsulated in a stable

Table 11.1 Classification of erythrocyte substitutes [6]

Classification		Modifications in preparations	Product (Company)	Prominent side effects in animal &/or human trials	Current status
Hemoglobin-based oxygen carriers (HBOCs)	Cross-link	α-α cross-linked (Fumarate) (human Hb)	HemAssist (Baxter, IL)	HTN, MI, CVA, higher mortality	Phase III halted in 1999
	Cross-linked & conjugated	Cross-linked & conjugated (human Hb)	PHP/Hemoximer (Apex bioscience)	HTN, ARF, death CVA, MI,	PHP: Phase II completed Abandoned in 2011
	Cross-linked & polymerized	Cross-linked & polymerized (human Hb)	Hemolink (o-raffinose) (Hemosol, Toronto, Canada)	HTN, severe cardiotoxicity, MI, CVA, TIA, high mortality	Phase III completed. Abandoned
			PolyHeme (Northfield Labs, Evanston, IL)	HTN, MI, CVA, TIA, ARF, higher mortality	Phase III completed in USA, but no FDA approval. Abandoned in 2009
	Polymerization	Glutaraldehyde polymerization (bovine Hb)	Hemopure (Biopure)	HTN, elevated liver enzyme, methemoglobinemia,oliguria	Phase III completed in trauma & cardiac surgery. Approved for human use in South Africa & Russia
			Oxyglobin (Biopure)		Designed & approved for canine use in US and EU
		Polymerized (zero-linked) (bovine Hb)	OxyVita (OXYVITA Inc. Windsor, NY)	Animal study ongoing	Preclinical trial ongoing Research ongoing
	HBOC Conjugated	Maleimide-PEGylated human Hb(oxy)	Hemospan (MP40x) (Sangart, San Diego, CA)	HTN, MI, CVA, TIA, ARF, high mortality	Phase II & III completed. Development shelved in 2015.
		PEGylated carboxy-Hb (bovine Hb)	Sanguinate (Prolong, South Plainfield, NJ)	Dizziness, lethargy, musculoskeletal adverse events	Phase II complete, phase III will not complete. Terminated. Research ongoing
		Pyridoxalated Hb polyoxyethylene conjugate (PHP)	Hemoximer (Apex inc.)	High mortality	Phase III terminated 2011 due to futility. Abandoned
	Encapsulation	LEAcHb	Dr. Chang in McGill		Research ongoing
	Natural Hb	Erythrocruorin	From earthworm Lumbricus terrestris		Research ongoing
		Polychaete Arenicola marina	HemO2Life by Hemarina, Brittany, France		Phase I ongoing Research ongoing

(continued)

Table 11.1 (continued)

Classification	Modifications in preparations	Product (Company)	Prominent side effects in animal &/or human trials	Current status
Perflurocarbon hemoglobin		Flusol-DA-20 (Green Cross,Japan)		Disapproved by side effects in USA
		Oxygent (Alliance, San Diego, CA)	Increased CVA	Discontinued in 2001 due partially to high cost
		Oxycyte (Tenax therapeutics)	Increase ICH & effects on immune system	Phase IIb done. Discontinued.
		PHER-O2 (Sanguine Corp)	As blood substitute	Research ongoing
		NVX-108 (NuvOx Pharma)	As radiation sensitizer, not blood substitute	Research ongoing
		Perftoran (Russian academy) as Vidaphor (FluorO2 Therapeutics)	Dizziness, kidney pain, hypotension, hyperemia, lung symptoms, ↑HR, ↓BP, ↑temperature, headache	Yes, in Russia & Mexico. Awaiting clinical trials in USA
Genetic engineering	Recombinant	Optro (Somatogen & Eli Lilly)	HTN, high mortality rate	Phase II completed. Development halted.
Cultured or artificial RBC	Artificial RBC	Biomimetic or nanotechnology rebuilding RBC		Research ongoing
	Cultured RBC	From stem cells		Research ongoing

HTN hypertension, *MI* myocardial infarction, *CVA* cerebrovascular accident (stroke), *TIA* transient ischemic attack, *ARF* acute renal failure, *RBC* red blood cell, *ICH* intracerebral hemorrhage, *HR* heart rate, *BP* blood pressure

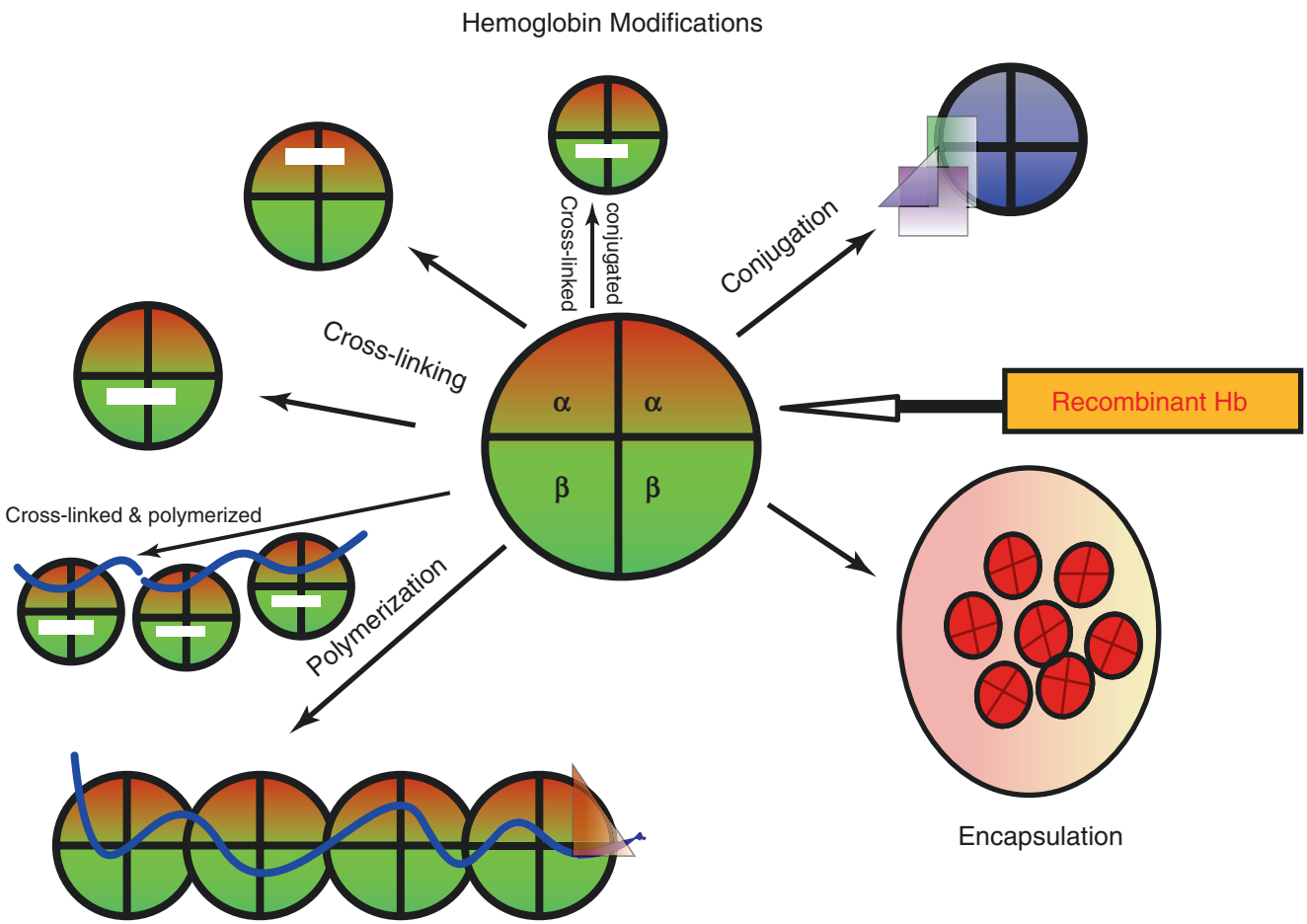


Fig. 11.2 Hemoglobin modification mechanisms

phospholipid bilayer capsule. In 1957, Chang et al. conducted the first experiment by microencapsulating hemoglobin molecules [24]. The phospholipid bilayer with cholesterol molecules functions as the RBC membrane, which provides increased rigidity and mechanic stability for liposome-encapsulated actin-hemoglobin (LEAcHb) [24].

Perfluorocarbon-Based Oxygen Carriers (PFBOC)

A biocompatible, non-blood, purely synthetic oxygen carriers has been a dream for numerous biomedical scientists in the last 80 years. Perfluorocarbons are chemically inert molecules similar to hydrocarbon structurally but the hydrogen groups are substituted with fluorine. Perfluorocarbon is 100 times smaller than erythrocyte. Perfluorocarbon molecules are emulsified in lipid solution due to its water insolubility [25]. The decades-long pursuit seemed to have shed some lights of hope at the end of the tunnel. However, it has proved to be an extremely daunting task to bring perfluorocarbon to the bedside for clinical patients. Without any doubt, perfluorocarbon is a fascinating molecule, it is the most hopeful chemical agent with great capacity to transport respiratory gases (oxygen and carbon dioxide). Researchers have made numerous modifications to the perfluorocarbon molecules to have formulated today's PFBOCs, which are very close to be used clinically in human patients. Perfluorocarbon molecules can carry oxygen and carbon dioxide without binding to these molecules [18], instead the oxygen molecules dissolve into molecular cavities within liquid droplets. The level of oxygenation in emulsified PFBOC is proportionately related to the partial pressure of oxygen in contact with perfluorocarbon molecules. The PFBOC infused into human circulatory system is removed by reticuloendothelial system and exhaled via the respiratory system [25]. PFBOC products today showed its potential applicability for many clinical indications [26–28]. There are several products belong to this category (Table 11.1). The four types of perfluorocarbons are as following:

- Perfluorodecalin: a decalin derivative with all of its hydrogen atoms are replaced by fluorine atoms. PHER-O2 belongs to this category.
- Perfluorotrypropylamine (PFTBA): an organofluorine compound from tributylamine with all the hydrogens being replaced by fluorine atoms, sometimes referred as FC43. Fluosol-DA is an example of PFTBA.
- Perfluorooctyl bromide: Perflubron is a synthetic radiopaque liquid form of perfluorooctyl bromide.
- Perflurorobutyl ethylene (PFBE): it does not have safety concerns documented in Europe.
- Perfluoro tert-butylcyclohexane: Oxycyte is in this category.

Oxygent, NVX-108, and Oxycyte also belong to this group. So far FDA only approved Fluosol DA-20.

Genetic Engineered Recombinant Hemoglobin

Genetic engineered hemoglobin by using recombinant DNA technologies to produce modified hemoglobin molecules in non-human organisms, such as E. coli and yeast [29]. When using these techniques, some amino acid sequences of natural human hemoglobin will be replaced to prevent disassociation into dimers and maintain oxygen affinity. Via a plasmid vector, the hemoglobin gene is transferred into E. coli cells, the gene expression of these transferred hemoglobin genes will induce the production of hemoglobin proteins in the E. coli. This technique does not have concerns related to spreading disease due to blood transfusion [30]. This technique however is not inexpensive, its high costs are major huddles for massive production. Optro is the first product in this category. Optro by Somatogen, Inc. and Eli Lilly is a genetically engineered variant of human hemoglobin, Hemoglobin Presbyterian.

Artificial and Cultured Red Blood Cells

Though for an artificial RBC to be almost the same as real human RBC is still a dream, recent developments in nanotechnology and other innovations have made this dream steps closer to the reality. Newer generations of artificial RBCs are all shining lights of hope to a complete erythrocyte substitute [31].

(a) Biomimetic Rebuilding of Multifunctional Red Blood Cells: Guo et al. reported a bioreplication approach based on a silica cell. They designed and constructed synthetically rebuilt RRBCs, which mimic the natural RBC properties in terms of size, deformability, biconcave shape, oxygen-carrying capacity, and even long circulation time. This process involves four steps: RBC bioreplication, layer-by-layer polymer deposition, precision silica etching, and RBC ghost membrane vesicle fusion [32].

(b) Artificial RBCs using nanobiotechnology
Some clinical conditions only require increased oxygen carrying to the tissues from the lungs. However, there are conditions that will require more than oxygen carrying. Thus, newer polyhemoglobins containing antioxidant enzymes are being developed. Innovations in lipid membrane artificial red blood cells and biodegradable polymeric nano- artificial red blood cells bring the dream and hope to our medical community and physiology again. Artificial RBCs using nanobiotechnology will likely function as oxygen carriers, oxygen carriers with antioxidant activity, and close to complete red blood cell substitutes [31, 33].

Of note, some literatures also termed encapsulated hemoglobin as artificial RBC, because the liposomal bilayer serves as the cell membrane wrapping around hemoglobin molecules.

(c) Cultured RBC
Stem cells have been successfully used in vitro to produce RBCs and researchers are currently challenged with developing larger-scale culture methods to meet the requirements for clinically relevant cell numbers. Recently, peripheral blood mononuclear cells were also successfully used for in vitro cultured RBCs. Additionally, these cultured RBCS can be customizable. Large-scale, cost-effective production depends on optimization of culture conditions [34].

Erythrocyte Substitutes Classification Based on Hemoglobin Sources

1. Human hemoglobin: basically, from those outdated stored human blood units
2. Bovine hemoglobin: from bovine blood, this can be massively produced.
3. Invertebrate natural hemoglobin
   (a) The Arenicola marina: $HemO_2Life$ is being developed by Hemarina in France.
   (b) The common earthworm Lumbricus terrestris: Erythrocuorin
4. Genetically engineered recombinant hemoglobin from E. Coli or yeast, this category also has the potential for mass production.

Erythrocyte Substitutes: Classification Based on RBC Membrane

1. Acellular hemoglobin molecules
   (a) Natural acellular hemoglobin: This category currently includes two sources of natural acellular hemoglobin: the Arenicola marina ($HemO_2Life$ is being developed by Hemarina, Brittany, France) and the common earthworm Lumbricus terrestris: Erythrocuorin molecules can carry oxygen molecules.
   (b) Natural human or bovine hemoglobin: The natural human hemoglobin is from the outdated human blood and bovine hemoglobin is from bovine blood products.
   - Polymerized Hb: Normal hemoglobin molecule is a tetramer of two α- chains and two β- chains. Polymerization of hemoglobin molecules can significantly increase the size acellular hemoglobin product, thus minimize the extravasation and prolong half-life in circulation. Polymerization mainly achieved with glutaraldehyde.
   - Cross-linked hemoglobin
     Cross-linking of hemoglobin monomer chains can prolong their half-life in the blood. These cross-linking can be between two α- chains or two β-chains. The linkers can be diaspirin or raffinose. It is believed that α-α chains cross-linking can prevent dissociation of oxyhemoglobin into αβ dimers which are readily excreted by the kidneys.
   - Conjugated hemoglobin
     Hemoglobin conjugation with the antioxidant enzymes can potentially protect hemoglobin molecules against free radicals.
   - Cross-linked and polymerized Hb
     Hemoglobin molecules can also be cross-linked and polymerized to further increase its half-life and/or minimize extravasations.
   - Encapsulated natural hemoglobin
     Hemoglobin molecules are wrapped with lipid layer(s) to provide protection against oxidative stress and prolong half-life.
2. The hemoglobin molecules can be encapsulated by liposomal lipid layers to extend their circulation half-life, improve their biodistribution and alleviate the commonly-encountered free-hemoglobin-induced toxicities. Cellular with artificial membrane
   (a) Biomimetic Rebuilding of erythrocytes
   With current biomimetic technology, it is possible to construct and synthetically rebuild RBCs that mimic almost the whole features of native RBCs in size, deformability, biconcave shape, oxygen-carrying capacity, and circulation time [32].
   (b) Artificial erythrocytes with nanotechnology
3. Cultured Erythrocytes
   Stem cells can be cultured to produce RBCs and peripheral blood mononuclear cells can successfully be cultured to reproduce RBCs as described previously.

Erythrocyte Substitutes: Classification Based on Organic Molecule

1. Organic: all hemoglobin-based oxygen carriers
   The oxygen-carrying molecules are biological molecules such as hemoglobin molecules, either natural or synthetic.
2. Inorganic: Perfluorocarbon-based oxygen carriers
   The oxygen-carrying molecules are not biological but inorganic chemical molecules, such perfluorocarbon-containing molecules.

Erythrocyte Substitutes: Classification Based on Synthetic or Natural Molecule

1. Natural molecule oxygen carriers
   This includes modified natural human or animal HBOCs, liposomal encapsulate Hb, Recombinant/transgenic Hbs, Natural acellular Hb from Lumbricus terrestris or Polychaete Arenicola marina, cultured erythrocyte, and albumin-heme hybrids.
2. Synthetic oxygen carriers
   This category includes PFBOCs, artificial erythrocyte, synthetic metal chelates, lipid-heme vesicles, and Hb aquasomes.

Ideal Erythrocyte Substitutes

An ideal red blood cell substitute should possess the following features [25]. However, none of the currently available products meets these criteria.

1. Great O2-carrying capacity: ≥ biological blood
2. Universal compatibility (no need crossmatch)
3. Pathogen-free (no blood-borne infection)
4. Long shelf-life, Survival of wide range storage conditions
5. Adequate supply, immediately/easily available
6. Minimal side effects, no/minimal toxicity
7. Volume expander
8. Reasonable half-life in circulation (Fig. 11.3)

Potential Indications for Blood Substitutes

The potential clinical indications of administration of blood substitutes include:

1. Trauma patients for volume replacement and stabilization [25].
2. Acute normovolemic hemodilution and perioperative volume replacement in major surgical procedures with potential massive blood loss.
3. Cardiovascular Surgery for pump priming, deep hypothermia and intraoperative blood replacement, or as cardioplegia.
4. Blood disorder management [35]
5. Oxygenation of solid tumors to increase its susceptibility to radiotherapy and chemotherapy
6. Ischemic tissues perfusion in patients with sickle cell disease, strokes, and peripheral vascular diseases
7. Organ preservation: During transport of organs for transplantation
8. Drug Carrier: the conjugated hemoglobin and perfluorocarbons can be used as drug carrier
9. Contrast Agent: Perfluorooctyl bromide can potentially be used as a contrast agent with oxygen carrying capac-

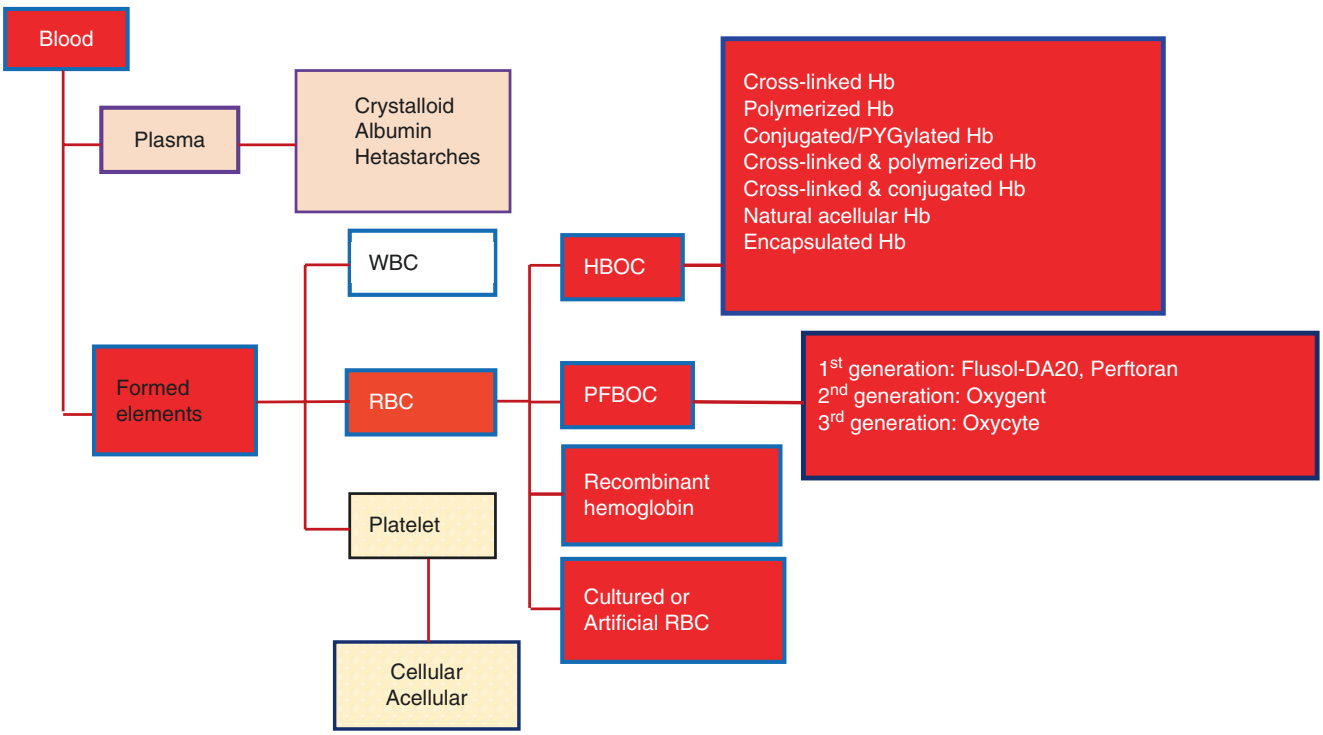


Fig. 11.3 Classification of the blood substitutes

ity in ultrasound, CT scan, MRI, angiography, liver, spleen and tumor imaging.
10. Miscellaneous: Anaerobic infections, gas embolism, CO poisoning.

Summary

The search for alternatives for allogeneic human blood has been ongoing for 80 years. There are numerous products at various stages. This is chapter described the different classifications of blood substitutes. Blood substitutes can be classified based on blood component into erythrocyte, leukocyte, thrombocyte, and plasma substitutes. Erythrocyte substitutes can be further classified as hemoglobin-based oxygen carriers, perfluorocarbon-based oxygen carriers, genetically engineered recombinant hemoglobin oxygen carriers, and cultured or artificial erythrocytes. Erythrocyte substitutes can also be classified based the source of hemoglobin, existence of cell membrane, organic or inorganic oxygen carriers, and synthetic or natural molecules.

Key Points

- Global shortage of allogeneic human blood supply will continue and likely worsen in the future.
- Only few products approved to be used clinically in limited regions (Hemopure, Perftoran) after 80 years, the accumulation of these efforts will usher in a breakthrough with bright future
- Blood substitutes can be classified based on blood components as erythrocyte, leukocyte, platelet and plasma substitutes.
- Erythrocyte substitutes can be classified as HBOCs, PCBOCs, Genetically-engineered recombinant Hb, and cultured or artificial erythrocytes.
- Erythrocyte substitutes can also be further classified based the source of hemoglobin, either organic or inorganic molecules, either natural or synthetic molecules,

Acknowledgements All authors have no conflict of interests.

References

1. Looker D, Abbott-Brown D, Cozart P, Durfee S, Hoffman S, Mathews AJ, et al. A human recombinant haemoglobin designed for use as a blood substitute. Nature. 1992;356:258–60. https://doi.org/10.1038/356258a0.
2. Sarkar S. Artificial blood. Indian J Crit Care Med. 2008;12(3):140–4. https://doi.org/10.4103/0972-5229.43685.
3. Cohn EJ. Blood: a brief survey of its chemical components and of their natural functions and clinical uses. Blood. 2015;126(24):2531. https://doi.org/10.1182/blood-2015-10-676718. PMID: 26635404.
4. Mathew J, Sankar P, Varacallo M. Physiology, blood plasma. [Updated 2020 Oct 27]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020. Available from: https://www.ncbi.nlm.nih.gov/books/NBK531504/.
5. Stanworth SJ, New HV, Apelseth TO, et al. Effects of the COVID-19 pandemic on supply and use of blood for transfusion. Lancet Haematol. 2020;7(10):e756–64. https://doi.org/10.1016/S2352-3026(20)30186-1.
6. Jahr JS, Guinn NR, Lowery DR, Shore-Lesserson L, Shander A. Blood substitutes and oxygen therapeutics: a review. Anesth Analg. 2021;132(1):119–29. https://doi.org/10.1213/ANE.0000000000003957. PMID: 30925560.
7. Armstrong SH. The management of blood preservation and blood substitutes. Bull N Y Acad Med. 1946;22(9):451–64. PMCID: PMC1871538.
8. West KA, Conry-Cantilena C. Granulocyte transfusions: current science and perspectives. Semin Hematol. 2019;56(4):241–7. https://doi.org/10.1053/j.seminhematol.2019.11.002. Epub 2019 Nov 8. PMID: 31836030.
9. Coller BS, Springer KT, Beer JH, Mohandas N, Scudder LE, Norton KJ, West SM. Thromboerythrocytes. In vitro studies of a potential autologous, semi-artificial alternative to platelet transfusions. J Clin Invest. 1992;89(2):546–55. https://doi.org/10.1172/JCI115619. PMID: 1737845; PMCID: PMC442886.
10. Wu D, Jin X, Wang X, Ma B, Lou C, Qu H, et al. Engineering temperature-sensitive plateletsomes as a tailored chemotherapy platform in combination with HIFU ablation for cancer treatment. Theranostics. 2019;9(13):3966–79. https://doi.org/10.7150/thno.32172. PMID: 31281525; PMCID: PMC6587342.
11. Safar P, Takaori M, Kirimli B, Kampschulte S, Nemoto E. Plasma substitutes for resuscitation. Prog Clin Biol Res. 1978;19:91–107. PMID: 78501.
12. Wiedermann CJ. Do plasma substitutes have additional properties beyond correcting volume deficits? Shock 25:2103-116, 2006. Shock. 2007;27(3):339–42. https://doi.org/10.1097/01.shk.0000258373.10244.5e. PMID: 17304118.
13. Amberson WR, Mulder AG, Steggerda FR, Flexner J, Pankratz DS. Mammalian life without red blood corpuscles. Science. 1933;78(2014):106–7.
14. Amberson WR, Flexner J, Steggerda FR, Mulder AG, Tendler MJ, Pankratz DS, et al. On the use of ringer-locke solutions containing hemoglobin as a substitute for normal blood in mammals. J Cell Comp Physiol. 1943;5:359.
15. Li S, Nickels J, Palmer AF. Liposome-encapsulated actin-hemoglobin (LEAcHb) artificial blood substitutes. Biomaterials. 2005 Jun;26(17):3759–69. https://doi.org/10.1016/j.biomaterials.2004.09.015.
16. Chang TMS. Hemoglobin corpuscles. J Biomater Artif Cells Artif Organs. 1988;16:1–9.
17. Liu L, Martínez JL, Liu Z, Petranovic D, Nielsen J. Balanced globin protein expression and heme biosynthesis improve production of human hemoglobin in Saccharomyces cerevisiae. Metab Eng. 2014;21:9–16. https://doi.org/10.1016/j.ymben.2013.10.010.
18. Hill SE. Oxygen therapeutics-current concepts. Can J Anaesth. 2001;48(4 Suppl):S32–40.
19. Martínez JL, Petranovic D, Nielsen J. Heme metabolism in stress regulation and protein production: from Cinderella to a key player. Bioengineered. 2016;7:112–5. https://doi.org/10.1080/21655979.2015.1126016.
20. Moore EE, Moore FA, Fabian TC, Bernard AC, Fulda GJ, Hoyt DB, Duane TM, Weireter LJ Jr, Gomez GA, Cipolle MD, Rodman GH Jr, Malangoni MA, Hides GA, Omert LA, Gould SA, PolyHeme Study Group. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. J Am Coll Surg. 2009;208(1):1–13. https://doi.org/10.1016/j.jamcollsurg.2008.09.023. Epub 2008 Nov 7. PMID: 19228496.
21. Chen JY, Scerbo M, Kramer G. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-

based oxygen carriers. Clinics (Sao Paulo). 2009;64(8):803–13. https://doi.org/10.1590/S1807-59322009000800016. PMCID: PMC2728196.
22. Batool F, Stutz C, Petit C, Benkirane-Jessel N, Delpy E, Zal F, et al. A therapeutic oxygen carrier isolated from Arenicola marina decreased P. gingivalis induced inflammation and tissue destruction. Sci Rep. 2020;10(1):14745. https://doi.org/10.1038/s41598-020-71593-8. PMID: 32901057; PMCID: PMC7479608.
23. Elmer J, Palmer AF, Cabrales P. Oxygen delivery during extreme anemia with ultra-pure earthworm hemoglobin. Life Sci. 2012;91(17–18):852–9. https://doi.org/10.1016/j.lfs.2012.08.036. Epub 2012 Sep 13. PMID: 22982347; PMCID: PMC3511863.
24. Chang TMS. Hemoglobin corpuscles. Report of research project for Honours B.Sc., McGill University, 1957.
25. Haldar R, Gupta D, Chitranshi S, Singh MK, Sachan S. Artificial blood: a futuristic dimension of modern day transfusion sciences. Cardiovasc Hematol Agents Med Chem. 2019;17(1):11–6. https://doi.org/10.2174/1871525717666190617120045.
26. Riess JG. Oxygen carriers ("blood substitutes")-raison d'Etre, chemistry and some physiology. Chem Rev. 2001;101:2797–919.
27. Jägers J, Wrobeln A, Ferenz KB. Perfluorocarbon-based oxygen carriers: from physics to physiology. Pflugers Arch. 2021;473(2):139–50. https://doi.org/10.1007/s00424-020-02482-2.
28. Riess JG, Le Blanc M. Solubility and transport phenomena in perfluorochemicals relevant to blood substitution and other biomedical applications. Pure Appl Chem. 1982;54:2383–406. https://doi.org/10.1351/pac198254122383.
29. Ishchuk OP, Martínez JL, Petranovic D. Improving the production of cofactor-containing proteins: production of human hemoglobin in yeast. Methods Mol Biol. 2019;1923:243–64. https://doi.org/10.1007/978-1-4939-9024-5_11. PMID: 30737744.
30. Funaki R, Okamoto W, Endo C, Morita Y, Kihira K, Komatsu T. Genetically engineered haemoglobin wrapped covalently with human serum albumins as an artificial O2 carrier. J Mater Chem B. 2020;8(6):1139–45. https://doi.org/10.1039/c9tb02184a. Epub 2019 Dec 16. PMID: 31840728.
31. Chang TM. From artificial red blood cells, oxygen carriers, and oxygen therapeutics to artificial cells, nanomedicine, and beyond. Artif Cells Blood Substit Immobil Biotechnol. 2012;40(3):197–9. https://doi.org/10.3109/10731199.2012.662408. Epub 2012 Mar 13. PMID: 22409281; PMCID: PMC3566225.
32. Guo J, Agola JO, Serda R, Franco S, Lei Q, Wang L, Minster J, Croissant JG, Butler KS, Zhu W, Brinker CJ. Biomimetic rebuilding of multifunctional red blood cells: modular design using functional components. ACS Nano. 2020;14(7):7847–59. https://doi.org/10.1021/acsnano.9b08714. Epub 2020 May 11. PMID: 32391687.
33. Chang TM. Blood substitutes based on nanobiotechnology. Trends Biotechnol. 2006;24(8):372–7. https://doi.org/10.1016/j.tibtech.2006.06.005. Epub 2006 Jul 11. PMID: 16815577.
34. Heshusius S, Heideveld E, Burger P, Thiel-Valkhof M, Sellink E, Varga E, et al. Large-scale in vitro production of red blood cells from human peripheral blood mononuclear cells. Blood Adv. 2019;3(21):3337–50. https://doi.org/10.1182/bloodadvances.2019000689. PMID: 31698463; PMCID: PMC6855111.
35. Zhang N, Wei MY, Ma Q. Nanomedicines: a potential treatment for blood disorder diseases. Front Bioeng Biotechnol. 2019;7:369. https://doi.org/10.3389/fbioe.2019.00369. PMID: 31850329; PMCID: PMC6892756.

12 Hemoglobin-Based Oxygen Carriers: Brief History, Pharmacology and Design Strategies, Review of the Major Products in Clinical Trials, On-Going Studies, and Coagulation Concerns

Jonathan S. Jahr, Kimia Roghani, Yll Buqa, Allen Rojhani, Preya Jhita, and Hae Won Kim

Abbreviations

°C	degrees centigrade
2-3 DPG	2,3 Diphosphoglycerate
CABG	coronary artery bypass graft
cADP	collagen and adenosine diphosphate, a reagent for PFA-100 measurement of platelet adhesion
cEPI	collagen and epinephrine, a reagent for PFA-100 measurement of platelet adhesion
CO	carbon monoxide
Cu	copper
DBBF	bis(dibromosalicyl) fumarate
dL	deciliter
EU	European Union
FDA	United States Food and Drug Administration
g	gram
g/dL	gram per deciliter
Hb	hemoglobin
Hb_4	Four strands of hemoglobin to form tetramer
HBOC	Hemoglobin-based oxygen carrier
HIV	Human immunodeficiency virus
IND	Investigational New Drug Application
kDa	kilodalton
L	liter
Lys	lysine
M	molar concentration
M101	HEMO2-life
MA	Massachusetts
MAL-PEG	maleimido polyethylene glycol
mg/dL	milligrams per deciliter
NO	nitric oxide
O_2	oxygen
PEG	Polyethylene glycol
PFA-100	Platelet Function Analyzer-100
PLP	pyridoxal-5′-phosphate
pRBC	packed red blood cells
r	recombinant
RBC	red blood cell
SFH	Stroma Free Hemoglobin
SOD	superoxide dismutase
TACO	Transfusion associated circulatory overload
US	United States
Zn	zinc
α	alpha
β	beta

J. S. Jahr (✉)
Department of Anesthesiology and Perioperative Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
e-mail: j.s.jahr@mednet.ucla.edu; j.s.jahr@ucla.edu

K. Roghani
University of Queensland-Ochsner Medical School, New Orleans, LA, USA
e-mail: k.roghani@uqconnect.edu.au

Y. Buqa
Dental Medicine, University Dentistry Clinical Center of Kosovo, Pristina, Republic of Kosovo
e-mail: yll.buqa@student.uni-pr.edu

A. Rojhani
Drexel University, College of Medicine, Philadelphia, PA, USA
e-mail: ar3493@drexel.edu

P. Jhita
Stanford University, School of Medicine, Stanford, CA, USA
e-mail: pjhita@stanford.edu

H. W. Kim
Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, School of Medicine, Providence, RI, USA
e-mail: hae_won_kim@brown.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_12

Introduction

Transfusion of erythrocytes (RBC) to treat acute or chronic anemia has significant drawbacks, given the risks of transfusion, volunteer donor requirements, limited supply with increasing demand, especially during a pandemic such as COVID-19, and erythrocytes are often unavailable in emergency situations or where blood is not an option. Significant work has been done for almost 100 years to attempt to replicate the functions of RBCs with oxygen carriers/oxygen therapeutics based on hemoglobin (Hemoglobin-based Oxygen Carriers, HBOC). This chapter will outline the background of HBOCs, discuss how HBOCs can be designed and how developed HBOCs are different from each other based on the changed pharmacology and physiology, highlight all major products to undergo human trials including one extensively studied product approved for human use in two countries (Hemopure), introduce newer products still under preclinical development, and finally present translational and clinical trials studying whether or not certain HBOCs may cause coagulation issues.

HBOCs have been extensively studied since 1934, when Amberson first used bovine hemoglobin and administered in canines, then in 1949 [1], he purified human hemoglobin and infused it into anemic patients. The U.S. Army developed a tetrameric cross-linked Hb that then was produced as the Baxter Corporation product, diaspirin cross-linked, although this product failed clinical trials because of increased morbidity and mortality [2], in the mid-1980s, a number of companies developed second generation HBOCs, including Biopure Corporation (Cambridge, MA) with Oxyglobin and Hemopure. Hemopure was approved by the Medicines Control Council in South Africa for treatment of anemia in 2001 and later in Russia and other countries, and Oxyglobin, FDA and EU approved for canine anemia in 1997, and 1998 respectively. To date, there have been a large number of studies evaluating these products in animal models 3–5 and clinical trials documenting success in phase I and II trials [3–6]. Hemopure was athe first to complete a Phase 3 trial (the other being PolyHeme,discussed below), while other products have undergone Phase 3 trials, but due to adverse events, were either terminated prematurely or suspended by regulatory agencies due to safety concerns.

Transfusion Medicine

Dating from 1795, blood transfusions rank with the most frequently utilized hospital procedures. In spite of the frequency of use, significant risk is linked: iron overload and Transfusion Related Acute Lung Injury (TRALI) [7]. Risk reduction related to improved blood banking techniques have mitigated infectious etiologies such as Chagas disease, HIV, Hepatitis C, malaria, *etc.*, issue with compatibility, and Transfusion Associated Circulatory Overload (TACO) [8].

Orthopedic surgical procedures, such as total joint arthroplasty and spinal instrumentation, have high perioperative transfusion rates [9], for the elderly, the perioperative blood loss and reduced tolerance for anemia increase the transfusion probability [10]. Blood use for patients older than 65 accounts for greater than 50% of perioperative erythrocyte transfusions and is predicted to double over the next 30 years [11]. Based on older data, a predicted shortfall of 4,000,000 units of blood in the US, more surgical procedures in an aging population, increasing blood use, and decreased donor blood collection will impact erythrocyte use across the country and the world [12].

Despite implementation strategies to conserve red cell use and the increasing safety of blood, the supplies of erythrocytes and components are unable to keep pace with the increasing demand [13].

Blood transfusion risks in addition to the diminishing supply of donated erythrocytes and blood products have mandated the requirement to develop artificial oxygen carriers (AOCs) and other substitutes for blood, including coagulation factors and platelets. If artificial oxygen carriers were able to be produced efficiently, and cost effectively, and be proven safe and efficacious, with long shelf life and stability, then these products would revolutionize medical care, both in the pre-hospital setting and beyond.

History of Hemoglobin-Based Oxygen Carriers

Amberson, in the 1930's was the first to document creation of HBOCs, and he tested his bovine hemoglobin in canine models and then human hemoglobin in parturients who had suffered hemorrhage and were unable to be transfused with erythrocytes [1]. Unfortunately, while all animals and humans improved transiently with the hemoglobin infusion, all died related to complications of renal failure due to blockage of renal tubules with dimers of hemoglobin, as is seen with all forms of hemolysis.

Early work involved lysing the red blood cell (RBC) coat to produce hemoglobin free of RBC membrane and structural stroma (SFH). These infusions of SFH led to jaundice, chemical pancreatitis and esophagitis, and ultimately renal failure. Toxicities of early generation HBOCs include renal failure, nitric oxide (NO) scavenging with resultant vasoconstriction, and hyper-oxidation engendering methemoglobinemia, while newer generation HBOCs aim to ameliorate these symptoms [14]. These toxicities appear due to the dissociation of the Hb tetramer into dimers, although changes in

Table 12.1 Synopsis of artificial oxygen carriers that have been tested in clinical investigations

Product	Company	Source	Modification	State of development
PEG-Hb	Enzon, Piscataway, NJ	Bovine	Polyethylene glycol-conjugated (PEGylated)	Phase lb, tumor radiosensitization, discontinued in 1996
HemAssist (DCLHb, diaspirin-crosslinked Hb)	Baxter, Deerfield, IL	Human	Intramolecular diaspirin α-α cross-linked tetramer	Phase III cardiac surgery, acute normovolemic hemodilution, trauma/stroke discontinued in 1999
Optro	Somatogen, Boulder, CO	Recombinant	Intramolecular cross-linked β-chain mutation (108 Lys)	Phase II discontinued in 1999
PHP/Hemoximer	Curacyte/Apex Bioscience, Triangle Park, NC	Human	Surface-modified polyoxyethylene-pyroxilated polymer	Phase III, distributed shock, discontinued in 2011
Oxygent	Alliance Pharmaceutical Corp, San Diego, CA	Chemical	Perfluorochemical emulsion	Phase III, discontinued in 2001
HemoLink (hemoglobin raffimer)	Hemosol, Toronto, Canada	Human	Intra- and intermolecular cross-linking with O-raffinose	Phase II/III, surgery, acute normovolemic hemodilution/cardiac surgery discontinued in 2004
PolyHeme (polymerized human Hb)	Northfield, Evanston, IL	Human	Glutaraldehyde polymerization	Phase III, trauma, surgery, discontinued in 2009
Hemospan (MP4)	Sangart Inc, San Diego, CA	Human	Maleimide-polyethylene glycol-modified Hb	Phase II published, phase III completed, discontinued in 2015
Hemopure (hemoglobin glutamer-250 [bovine])	Acquired by Hemoglobin Oxygen Therapeutics in 2014, Souderton, PA	Bovine	Glutaraldehyde polymerization	Phase III, perioperative transfusion, acute normovolemic hemodilution cardiac surgery. Hb glutamer-250 (bovine) approved for perioperative treatment of anemia in adult elective surgical patients in South Africa and Russia. Available for expanded access in the United States
Sanguinate	Prolong Pharmaceuticals, South Plainfield, NJ	Bovine	Polyethylene glycol-conjugated (PEGylated) carboxyhemoglobin	Phase II trials complete Phase III trial not complete
$HemO_2Life$	Hemarina, Morlaix, Brittany, France	Marine Invertebrate	Hexagonal-bilayer-linked globin molecules	Phase I in progress
OxyVita Hb	OXYVITA Inc, Windsor, NY	Bovine	Hb stabilized with sebacoyl diaspirin	Preclinical trials in progress

Adapted and modified from Jahr et al. [14]

the chemical molecular configuration of Hb, and issues with the microcirculation have also been suspect. Modifications such as cross-linkage, polymerization, and polyethylene glycol (PEGylation) conjugation, are all techniques that have been utilized in an attempt to diminish these toxicities and are reviewed in the section on "How HBOCs Are Made" [14]. See Table 12.1: Synopsis of HBOCs that have been Tested in Clinical Investigations [14].

How HBOCs Are Made

Stroma Free Hemoglobin

One of the early methods of isolating hemoglobin involves the creation of stroma-free hemoglobin (SFH). This is essentially purified hemoglobin isolated from human red blood cells (RBCs) free of the erythrocyte membrane and structural fragments (stroma). There are multiple ways to achieve this, one of which involves crystallization of hemoglobin. With crystallization, RBCs are lysed with water and toluene, centrifuged, and washed with a phosphate buffer. Hemoglobin crystals are then formed without the red cell membrane [15]. SFH can also be isolated with a commercially available blood cell separator. Using this motor-operated separator, donor blood is centrifuged to separate the plasma and isolate just the RBCs. The RBCs are then lysed by hypotonic shock and the stroma is separated, leaving just the Hb lysate [16]. Lastly, selective DEAE-cellulose absorption can be used to create SFH. Outdated RBCs are washed with saline, centrifuged at 5000 rpm at 4 °C, hemolyzed with distilled water for 30 minutes, then centrifuged at 5000 rpm at 4 °C for 30 minutes again. The lysate mixture is mixed with DEAE-52, then separated by vacuum filtration and dialyzed with a standard kidney dialysis buffer. The SFH is then isolated by pressure filtration [17].

α-α Cross Linked Hb

Normal hemoglobin in animals and humans is a tetramer, Hb4, comprised of two identical α chains and two identical β chains which are non-covalently bonded. It is possible to cross link the two individual alpha units of Hb in order to prolong its half-life in the body. The advantage of cross-linking the two alpha chains is because this alpha-alpha cross-link prevents dissociation of oxyhemoglobin into $\alpha\beta$ dimers which are readily excreted by the kidneys [18]. Snyder et al. found that the half-life of HbXL99 (α-α cross-linked Hb) in rats was increased to 3.3 hours compared to 90 minutes for α Hb alone. In order to cross link the two alpha chains, a commonly used cross-linking agent is bis (dibromosalicyl) fumarate (DBBF). To start, stroma-free hemoglobin is used and must initially be deoxygenated. Deoxygenation is achieved by reacting the Hb with a reducing agent such as sodium dithionite or ferrous citrate for 1–3 hours in room temperature. A cross linking agent, preferably DBBF is then added. DBBF has a high specificity for the Lys 99 residues on each of the Hb α chains. The newly α-α cross linked Hb can be separated from any unreacted Hb using chromatography. This α-α cross linked Hb is now resistant to alpha-beta dimerization and renal elimination [19].

Glutaraldehyde Polymerized Hbs

Hemoglobin can be polymerized with glutaraldehyde to create a large HBOC with less risk of extravasation and subsequent nitric oxide scavenging. Two notable HBOCs that are created using this method are Hemopure (HBOC-201), and Polyheme. Hemopure is a purified bovine Hb polymerized with glutaraldehyde for stability and formulated in a lactated Ringer's solution. PolyHeme is synthesized utilizing outdated human erythrocytes, modified with pyridoxal-5′-phosphate (PLP), and is then polymerized with glutaraldehyde [14]. As a whole, glutaraldehyde solutions are made up of polymers that react with hemoglobin at multiple cross-linking sites and create a heterogenous mixture of Hb. The process of glutaraldehyde polymerization involves a redox potential decrease, and an iron autoxidation rate increase [20]. To create soluble polymers, a solution of 0.05 M phosphate buffer (pH 6.8) is prepared along with 100 mg/mL of human oxyhemoglobin and 3.3 mg/mL of glutaraldehyde at 20 °C. After 20 minutes, glycine is added, and the solution is dialyzed with a phosphate buffer. The process by which insoluble polymers are created is as follows: a 0.05 M phosphate buffer (pH 6.8) is combined with 100 mg/mL of Hb and 3.3 mg/mL of glutaraldehyde. The solution is then frozen at – 30 °C for 2 hours and warmed slowly at 4 °C. The insoluble foam is then rinsed with a glycine solution and phosphate buffer.

Recombinant HBOC

Acellular recombinant hemoglobin can be used an oxygen carrier. Similar to HBOCs constructed from human or animal Hb, recombinant HBOCs (rHBOCs) potentially can be infused in place of erythrocytes. Recombinant Hb has the advantage that it can be mass produced utilizing molecular biology in unlimited amounts and does not require human Hb. Recombinant Hb can be produced in E. Coli by fusing alpha or beta globin cDNAs to the coding region of a bacteriophage. The fusion protein can then be recovered and refolded to produce tetrameric Hb [21]. A similar, newer, system involves incorporating the alpha or beta globin genes as a transcript in the tac promoter. Then, functional Hb tetramers are produced after exogenous heme is incorporated into the E. Coli cytoplasm [22].

Zero-Linked HBOCs

Another type of polymerized hemoglobin is zero-linked Hb. This is created by a procedure where hemoglobin's surface is activated with carbodiimide at the carboxyl groups. This causes the carboxyl groups to create covalent bonds to amino groups of an adjacent hemoglobin molecules. The name zero-link is derived from the fact that no chemicals are left behind in the linked polymers, unlike with glutaraldehyde or other polymerization techniques [23].

Pegylated HBOCs

PEGylated hemoglobin refers to the addition of polyethylene glycol to hemoglobin. Two important products that use pegylated Hb are PHP (Hemoximer), Hemospan and Sanguinate. The overall procedure involves deriving hemoglobin from bovine or outdated human sources and PEGylating it. Then, either maleimide is added to produce the Hemospan (MP4) product, or the Hb is PEGylated and carbon-monoxide hybridized to create Sanguinate [7]. Hemoglobin can be pegylated in two ways: or under anaerobic conditions to produce PEG-Hb(deoxy), or under aerobic conditions to produce PEG-Hb(oxy). To produce PEG-Hb (deoxy), a buffered solution of 50 mM sodium phosphate, and 100 mM KCl, 0.5 mM EDTA is placed in a 1 L bottle of pH 7.0 at 20 °C. Inositol hexaphosphate is added, Hb is reacted with iminothiolane, and then maleimido-polyethylene glycol (MAL-PEG) is added to the mixture. The solution is

dialyzed with a phosphate buffer saline to eliminate any unreacted MAL-PEG and passed through a filter to remove endotoxins. To produce PEG-Hb (oxy), PEG conjugation is done aerobically in a 1 L bottle at 5 °C. 3 mM Hb is treated with IMT in a phosphate buffer saline at pH of 7.4 for 4 hours, and then MALPEG is added. After an incubation period of 2 hours, a cysteine solution is added to terminate the reaction. The sample is passed through a syringe filter to remove endotoxins [24].

Invertebrate Hb

Hemarina's multiple products, including HEMO2-life, is derived from a large extracellular hemoglobin molecule that is naturally found in the invertebrate Arenicola marina, commonly known as the lugworm. Each Hb molecule from this species can transport 156 oxygen molecules, compared to just 4 oxygen molecules carried by human Hb [25]. In addition, HEMO2-life can function in the temperature range of 4–37 °C, and its size is 250 times smaller than a human RBC [26]. These properties give HEMO2-life remarkable utility for oxygen delivery in humans. A team of researchers led by Lupon et al., in France investigated optimal ways of packaging and delivering HEMO2-life. Using their technique, it is packaged in a 20 mL solution containing 1 g of active extracellular HEMO2-life, 203.3 mg of Magnesium chloride, 105.2 mg of sodium chloride, 100.3 mg of sodium gluconate, 73.5 mg of sodium acetate, 7.5 mg of potassium chloride, 7.3 mg of calcium chloride, 35.2 mg of ascorbic acid, and 20 mg of water for injection. The investigators found the half-life to be 48–72 hours and that the washout period maximum is 4 days. The team led by Alix et al., studied the addition of HEMO2-life (M101) to preserve liver grafts. The investigators discovered that when added to a cold storage solution liver graft, M101 effectively oxygenates the grafts during preservation, preventing post-transplant injury. The team also reported that M101 has intrinsic Cu/Zn superoxide dismutase (SOD) activity, likely contributing to its utility for preventing post-transplant injury.

Pharmacology and Physiology of HBOCs

Table 12.2 shows the pharmacology and physiology of HBOCs.

Development of HBOCs

As mentioned previously, the first generation of blood substitutes were the SFH products. SFHs were manufactured utilizing ultrafiltration or crystallization. The SFH prepared by ultrafiltration method was reported to lack the vasoconstriction and ex vivo perfused myocardial depressant factors [7]. However, SFH was later found to elicit many adverse effects to progress forward, but the untoward outcomes were valuable in determining the cause of the adverse effects.

Using the feedback from the SFH experiments, it was hypothesized that crosslinking may help reduce the adverse effects seen in SFH. Researchers had been working towards crosslinking hemoglobin since the mid-1960s and that effort was accelerated during the 1980s. At that time, the HIV epidemic was beginning, and the military was concerned the epidemic would deplete the blood supply for transfusions. Blood availability for transfusions were essential to treating wounded soldiers, and so a viable blood substitute became an urgent unmet need.

Development of αα-Hb and HemAssist

The group of scientists from the Army Institute of Research at Letterman (LAIR) were successful in creating a crosslinked Hb that could reduce the renal toxicity issues seen in SFH [28]. To be able to mass produce, LAIR contracted out to Baxter Healthcare. Together, they successfully crosslinked the α chains of hemoglobin utilizing bis(dibromosalicyl) fumarate (DBBF) [28]. The army called the product DBBF-Hb and ultimately $\alpha\alpha$-Hb, while Baxter (Deerfield, IL) referred to the product as diaspirin-crosslinked Hb (DCLHb), with the trade name of HemAssist. The relationship between LAIR and Baxter however was not collaborative as Baxter's focus was on increasing production while LAIR was focused on resolving vasoconstriction effects and other biological problems. Following a study on a pig model simulating a battlefield injury, LAIR stopped attempting to produce a blood substitute. LAIR declared that based on the pig model, $\alpha\alpha$-Hb was extremely vasoactive and therefore too toxic to be considered as a blood substitute option [28].

This decision was validated again by further studies showing systemic and pulmonary hypertension as well as doubling of the vascular resistance which was equivalent to that seen with inhibition of nitric oxide synthesis [29]. Baxter however continued with production of HemAssist and went on to be tested in human clinical trials because in their application to the Food and Drug Administration (FDA), Baxter explicitly stated that their product, HemAssist, was critically different from DBBF-Hb by LAIR [28]. Ultimately, HemAssist failed as well for being too vasoactive, just like DBBF-Hb.

In the critical study of HemAssist published by Saxena et al., two major adverse events were identified. One patient experienced transient renal and pancreatic insufficiency while the other suffered from both a fatal brain edema as well as a pulmonary edema [30]. Similar results were seen in

Table 12.2 Pharmacology and physiology of HBOCs

Product	HemAssist	HemoLink	Hemoximer	PolyHeme	Oxyglobin	Hemopure	Hemospan	OxyVita	Sanguinate	Hemarina
Production method	Alpha-alpha crosslinked Hb	Polymerized human Hb, cross-linked with o-raffinose	Pyridoxalated Hb polyoxyethylene conjugate	Glutaraldehyde polymerized human Hb	Acellular polymerized Hb	Purified cell-free glutaraldehyde cross linked and polymerized	PEGylated Hb plus maleimide	Zero-linked polymerization	PEGylated carboxy-Hb	Derived from large extracellular Hb molecule
Source	Outdated human Hb	Outdated human Hb	Outdated human Hb	Outdated human Hb	Bovine	Bovine	Outdated human Hb	Bovine	Bovine	Arenicola marina
Hb concentration (g/dL)	10	10	8	10	13	13	4.2	6	4–5	4
P50 (mmHg)	32	39 ± 12	23.4	20–22	34	40	6	6.4	7–16	7.05 ± 0.93
Hill coefficient	2.9	1.0 ± 0.2		1.7	1.3	1	1.2	1.2		
Viscosity (cp)	1.2	1.1	3	1.9–2.2	2.8	1.3	2.5	2.8		1.23
COP (mmHg)	42	25.4	49	20–25	42	25	55	2.2		1
pH	7.4	7.5 ± 0.5	7.4		7.8	7.6–7.9	7.5 ± 0.2	7.5	8	6.5–7.5
Solution		LR			LR	LR	LR	LR	Phosphate buffered saline	Injectable buffer
Half-life in humans (hr)	6–12	15–18		24	18–43	19	20–36	72	7.9–13.8	48–72
Stability/ storage	<5 °C for 1+ yr	Oxy-ligand form: −80 °C De-oxy-ligand form: 4 °C		4–8 °C: 1+ yr	Room temp: 3 yr	Room temp: 3 yr		Lyophilized: 5 yr. Liquid: 1 yr. at room temp	48 °C: 4 yr., Room temp: 2 yr	-20 °C ± 5 °C/−80 °C ± 10 °C, for 5 years
Molecular weight (kDa)	64	>100	109	150	200	250	90	33,000	109–120	3600

Adapted and modified from Reference [27] and from personal communication February 2021 with:
HemAssist: Dr. Tim Estep, Hemolink: Dr. Davy C.H. Cheng, Hemoximer: Dr. Joe DeAngelo, PolyHeme: Dr. Eugene Moore & Dr. Alexis Craloey, Hemopure: Dr. Greg Dube', Hemospan: Dr. Peter Keipert, Sanguinate: Dr. Abe Abuchowski, OxyVita: Dr. Hanna Wollocko, Hemarina: Drs. Franck Zal and Eric Delpy

the study conducted by Sloan et al. in which 46% of the patients given HemAssist died 28 days after administration compared to the 17% who received only saline [31]. This failure by Baxter placed further emphasis on preclinical testing prior to administration to human subjects.

Development of Hemolink

Prior to the cessation of investigations of HemAssist, a second generation of HBOCs were developed, the earliest being Hemolink, made by Hemosol Inc. (Mississauga, ON, CA). Hemolink, from outdated human blood hemoglobin, is a raffinose crosslinked HBOC [27]. An indicated use was for cardiac surgery and achieved human clinical trial status. In a phase II clinical trial using the intraoperative autologous donation (IAD) concept during cardiac surgery, patients given Hemolink required less allogeneic erythrocytes, even past the operative period [32]. In another phase II dose escalation trial conducted by Hill et al., Hemolink was successful at reducing allogenic red cell transfusion for subjects having coronary artery bypass graft (CABG) surgery [33].

Hemolink clinical trials were stopped voluntarily during a Phase IIb trial due to concern over increased morbidity and mortality [27, 34]. There was a disproportional incidence of adverse cardiac events in the group administered Hemolink, although the higher occurrence of adverse events may have been due to the increased number of diabetics within the Hemolink treated group [34]. Hemolink was created with the rational that HBOC s would restore the oxygen carrying capacity of the blood lost and ultimately facilitate an increase in blood pressure [35]. However, although vasoconstriction does increase central blood pressure, it may, by the same pathway, decrease downstream capillary pressure, and might be responsible for causing myocardial ischemia.

Development of Polyheme

A reoccurring issue with HBOCs was the vasoconstrictive properties they exhibited due to the vasoactive tetramers. Researchers hypothesized the vasoactive tetramer properties were due to trans-endothelial extravasation of the small molecular weight tetramer, which lead to abluminal binding of nitric oxide (NO) and induced unrestricted vasoconstriction [36].

To alleviate extravasation, Northfield Laboratories produced a nearly tetramer-free and considerably larger HBOC from glutaraldehyde polymerized human Hb. PolyHeme's polymerization technique improves intravascular persistence and is then purified until nearly all unpolymerized tetramers are removed. This reduces interactions with nitric oxide and therefore potentially reducing vasoconstriction [36].

PolyHeme was hypothesized to have indications as a resuscitation fluid, especially helpful in avoiding the onset of anemia and delaying the inevitable mortality until surgical intervention and red cell transfusion would be available [27]. PolyHeme was designed as an asset for shock and organ ischemia, especially in emergencies.

Early clinical trials suggested PolyHeme was successful in providing oxygen-carrying capacity at life-threatening Hb levels and maintaining oxygen transport during intense blood loss. With these results, the FDA approved a Phase III clinical trial. This pivotal multicenter trial published by Moore et al. was significant due to its clinical results but also for the ethical issues that arose. The protocol was centered on two hypotheses regarding survival benefits: early replacement of oxygen-carrying capacity in a setting in which blood is not available, and PolyHeme administration in place of blood transfusion during the first 12 hours after injury to decrease the immunoinflammatory response and ensuing organ dysfunction [36].

The study enrolled over 700 patients and was divided into two phases, the "pre-hospital" and "in-hospital". During the pre-hospital phase, incapacitated trauma patients were either given the standard of care (saline) or given PolyHeme. In the in-hospital phase, the subjects administered saline in the pre-hospital phase then were administered erythrocytes (allogeneic), whereas subjects who were given PolyHeme in the pre-hospital phase continued with PolyHeme administration, instead of the standard of care administration of allogeneic blood. While the patients given PolyHeme required and were administered fewer units of erythrocytes, more PolyHeme cohort subjects were also designated as having "probable myocardial infarction" [36].

The study was discontinued in Phase III following negative results [27]. The ethical issues stemmed from the portion of the trial occurring post hospital admission. Since 1996, the FDA had published regulations that waive the requirements for informed consent in specific emergency research protocols. Researchers in this clinical trial did not therefore need to obtain personal consent once the subjects arrived at the hospital prior to administration of PolyHeme. Once at the hospital, blood transfusion is the standard of care for trauma patients; however, these subjects were not able to consent to waive the erythrocytes (standard of care) and instead administered an experimental protocol.

Development of Hemoximer

See Table 12.3 for development of Hemoximer (PHP).

Table 12.3 Development of Hemoximer (PHP)

Compelling evidence suggests that NO is the causative agent responsible for vasodilation and hypotension in distributive shock. In the successfully conducted Phase II clinical development program PHP has previously been demonstrated to reverse vasodilation and resolve hypotension associated with this type of shock
The PHOENIX trial was a European, placebo-controlled, Phase III study treating patients in catecholamine-resistant, distributive shock with the Nitric Oxide (NO) scavenger PHP/Hemoximer.
This phase III, multi-center, randomized, placebo-controlled study compares the effectiveness of continuous infusion of PHP/Hemoximer plus conventional vasopressor therapy against placebo (normal saline) plus conventional vasopressor therapy in patients with catecholamine-resistant distributive shock. In addition, the safety and tolerability of this new treatment modality will be evaluated.
For inclusion into the trial the patients had to be adequately resuscitated with fluids and must require a norepinephrine dose of ≥0.3 mcg/kg/min to maintain a mean arterial blood pressure of ≥65 mmHg. Furthermore, patients had to fulfill at least two criteria indicative of a systemic inflammatory response ("SIRS" criteria). PHP/Hemoximer as active compound or placebo was administered by continuous intravenous infusion at 0.25 mL/kg/hr. for a maximum of 150 hours.
Efficacy was to be demonstrated by PHP significantly reducing 28-day all-cause mortality. Secondary endpoints include: Survival time, survivor days in the intensive care unit ("ICU") and time on mechanical ventilation and on vasopressors.
The study was launched by Curacyte AG in Austria, Belgium, Germany, Spain, The Netherlands and in the United Kingdom in 2009. The outcome of the third interim analysis on safety and mortality data of 300 patients, or 66% of the study population in the trial is reported. Statistical results were reviewed in an unblinded fashion by an independent Data Monitoring Board (DMB). The DMB, consisting of intensive care physicians, one bioethical expert and one statistician from the US and Europe came to the unanimous conclusion, that the study should be terminated.
The trial was designed to statistically prove survival benefit of patients treated with PHP after 28 days compared to placebo (+ standard care), and at day 60 and day 90 as secondary endpoints. After treatment of two thirds of the patient population the numerical number of deaths in the PHP/Hemoximer cohort exceeded that of the placebo group. Given the current status of the study there is no more chance that the study could demonstrate a statistically significant efficacy on 28-day all-cause mortality in favor of PHP/Hemoximer. Therefore, the continuation of the PHOENIX study with enrolment of new patients under the premise to detect an unlikely difference would be unethical. Based on the DMB's recommendation Curacyte AG suspended the PHOENIX trial.

Reprinted from: vm-lifescience.com/curacyte-phase-iii-distributive-shock-trial-phoenix-trial-terminated-for-futility/ Accessed 2.16.2021

Development of Hemopure and Summary/ Discussion of Pivotal HEM-0115 Trial

The final second generation HBOC product to be discussed is Biopure's Hemopure (HBOC-201), one of the most extensively studied HBOCs (now produced by Hemoglobin Oxygen Therapeutics, Souderton, PA, USA). Hemopure's hemoglobin is extracted from bovine RBC, purified and then polymerized with glutaraldehyde, after extensive cleaning processes, validated by the US FDA to ensure removal of bacteria, fungi, parasites, viruses and prions. Hemopure draws its benefit from maintenance of oxygen delivery with anemia or low blood flow situations to ischemic tissues. Unlike its other fellow second generation HBOCs, Hemopure has gained regulatory indication for human use in South Africa (2001), and in Russia (2006) to treat acute surgical anemia.

Hemopure is a glutaraldehyde cross-linked, polymerized bovine hemoglobin that is cell free and purified positioned in a changed lactated Ringer's solution with 13.6 g/dL Hb (30–35 g Hb/250 mL unit), a pH of 7.6–7.9, and a P50 of 40 mmHg. Hemopure may be kept at 20 degrees centigrade for up to 3 years, is not in need of cross-matching, has an oxygen release that is independent of 2,3-diphosphoglycerate, and has a half-life of 19 hours in the circulation [37].

Drawing from four different studies in which RBC transfusion was used as the control, Hemopure administration led to lower allogeneic RBC units transfused [27]. In a randomized and double-blind trial conducted by Levy et al., HBOC-201 was evaluated for its treatment of moderate anemia following cardiac surgery due to blood and hemodilution. In the study, 98 patients scheduled to cardiac surgery were randomly divided into the control group, set to receive RBC transfusion, and the other to receive HBOC-201, and then monitored during the course up to their third postoperative transfusion. Of the 50 patients given Hemopure, 17 of them did not require any RBC transfusion following the operation [5]. An important point is that although this study concluded that Hemopure administration decreased the requirement for transfusion of erythrocytes postoperatively in one third of its participants, the study was not designed to compare the mortality and morbidity and so no conclusions were able to be reached with regard to its efficacy relative to blood.

A more detailed description of the HEM-0115 study follows [37].

The research study evaluated Hemopure as an alternative blood transfusion and its safety and efficacy in a Phase III, single blind, multicenter trial in elective orthopedic surgery. The whole trial study population included male and female (nonpregnant/lactating women) subjects, 18 years or older. The investigators randomized six hundred eighty-eight patients to treatment with Hemopure or erythrocytes. Paired/matched group analysis was performed comparing groups directly, determined by a predetermined prospective dichotomy (success of treatment compared to failure in the Hemopure arm and similarly predetermined dichotomy in the erythrocyte arm, determined by the requirement for additional oxygen carrying capacity [moderate compared to high]). The hypothesis

was to prove the ability of Hemopure to safely reduce or eliminate perioperative transfusion.

In the majority of subjects, Hemopure administration demonstrated the eliminate of need for erythrocytes. Comparing the Hemopure and erythrocyte arm in a safety analysis demonstrated disequilibrium and was linked to subject's age, volume overload, and under treatment, and occurred in those subjects in whom Hemopure alone was insufficient. The investigators concluded that in subjects under 80 years of age with a clinical requirement deemed moderate, may be infused with up to 10 units of Hemopure to avoid erythrocytes safely.

This landmark, pivotal study aimed to demonstrate efficacy, safety and adverse event profile of HBOC-201 use. Treatment with Hemopure exceeded the pre-determined primary endpoint of 35% avoidance of transfusion with an overall 59% avoidance observed at the 6-week follow-up assessment.

One of the most interesting evaluations for the management of low hematocrits is whether the transfusion criteria is relevant to current transfusion practice. The study demonstrates that this clinical decision must be tempered by the patient's clinical status with respect to cardiac disease, pharmacologic agents, and a host of other factors [38, 39].

The concept of a universal transfusion trigger has fallen out of favor with the recognition that the decision to transfuse is actually quite complex, based upon individual patient factors beyond total Hb concentrations as reflected in the most current United Kingdom (2007) and United States (2006) guidelines [40, 41]. The term transfusion "threshold" is now preferred.

The results of matching group and root cause analyses suggest that the future use of Hemopure in patients over age 80 and/or when the need for additional oxygen carrying capacity exceeds what can be met with Hemopure alone should be limited to when blood is not available. Vigilance to patient vascular volume status is required when infusing Hemopure; avoidance of fluid overload and, if present, aggressive management is required. Patients with preexisting cardiac disease are less tolerant of lower total Hb concentrations and may be at greater risk of morbid events and increased mortality.

The most robust observations have come from large retrospective studies which suggest that for older patients and patients with cardiac disease relatively small deviations from normal Hb and Hct levels may be associated with an increased risk of morbidity and mortality [42, 43].

In the Hemopure arm, more than 59% of subjects did not require erythrocytes. Consequences deemed adverse and those deemed serious (serious adverse events [SAEs]) per subject were greater in the Hemopure versus erythrocyte groups. Volume overload and undertreatment may explain this difference.

Transfusion was eliminated in the Hemopure arm in most subjects. The safety analysis of the two different study groups (H vs. R) did not reach favorable status probably due to subject's age, hydration status and possible volume overload, and potential under administration of Hemopure, and was isolated to subjects who were unable to be cared for only with Hemopure. Nonetheless, octogenarians with modest acute anemia safely may avoid transfusions when managed with up to 10 units of Hemopure.

Adverse Events of Particular Interest

The vasoactivity of HBOCs as a drug class has been of great general interest, with some HBOCs showing greater vasoactivity than others [44]. Blood pressure elevations were transient with mean SBP [Systolic blood pressure] 10–15 mm Hg above that observed with PRBCs.

No significant changes were noted in the cardiac biomarker CK-MB between treatment arms, differences in troponin elevations were observed; with 5 of the 18 HBOC-201 patients with elevations of troponin showed serial elevations.

The analyses described in the 'Study Limitations and Critique' section suggest that when the Schedules for Maximum Surgical Blood Orders predicts a "need" of three units of erythrocytes or less, in the patients under 80 years old who are undergoing elective orthopedic surgical procedures, safe, and effective avoidance of red cell transfusion is possible with the use of up to 10 units of Hemopure. Whenever erythrocytes are not an option, treatment with Hemopure may be appropriate and might be optimal.

In conclusion, the HEM-0115 Phase III study demonstrated Hemopure's reduction or elimination of erythrocyte requirements in patients undergoing orthopedic surgery. Six hundred, eighty-eight subjects were randomized to be administered Hemopure or erythrocytes. For up to 6 days, Hemopure subjects were infused with a maximum of 325 g/2500 mL of Hemopure. Erythrocytes were then transfused as needed. Hemopure's reduction of the requirement for more erythrocytes occurred in more than 59% of the subjects. The Hemopure cohort reported an increased rate of adverse effects that consider was significantly greater [45].

Recent Clinical Trials on Hemopure

In a study examining the preoperative therapeutic effects of Hemopure, LaMuraglia et al. concluded preoperative

Hemopure administration to anemic patients undergoing aortic surgery decreased the need for erythrocytes in almost one third of the patients [46]. Based on the two studies, Hemopure administration before or after helped alleviate the need for RBC transfusion. The FDA has yet to approve a trial testing Hemopure's efficacy on trauma patients due to apprehensions regarding trial design and study justification [45]. As discussed earlier, there were significant ethical concerns raised over the trial design of Polyheme's use in trauma settings and further trauma trials will need thoroughly designed protocols to receive approval.

However, recently, the US Department of Defense approved and funded a trauma trial to be conducted in South Africa (https://www.dvidshub.net/news/printable/311421 accessed 1/31/2021) with Hemopure. See Table 12.4 describing the plans for the study.

Table 12.4 Plans for the Hemopure Trauma Trial in South Africa

Experts from the US Army Institute of Surgical Research and Emergency Medicine and South Africa's Stellenbosch University (SU) have embarked on a large, multi-institutional clinical trial to evaluate the use of synthetic blood-products for the resuscitation of trauma victims before they arrive at a hospital.
The Institute of Surgical Research is coordinating through the US Africa Command Science department and under an agreement implemented by the US Embassy Pretoria, Office of Defense Coordination on Research, Development, Testing and Evaluation (RDT&E) in 2016.
The San Antonio based center, which focuses on combat casualty care, will be partner with Stellenbosch University's division of emergency medicine Faculty of Medicine and Health Sciences.
Other universities involved in the clinical trial include the University of Cape Town, KwaZulu-Natal, Witwatersrand and Pretoria. These entities collectively represent the body of academic emergency medicine in South Africa.
All parties met in Cape Town for the first major planning session 27–30 November at Stellenbosch University's Tygerburg multi-center, prospective, randomized clinical study of bioplasma freeze dried plasma and Hemopure for use in treatment of trauma patients with significant Haemorrhage.
The study will evaluate the use of the hemoglobin-based oxygen carrier Hemopure (HbO2 therapeutics LLC) together with bioplasma FDP (National Biologics Institute), freeze-dried plasma, to resuscitate trauma victims prior to arrival at a hospital emergency room.
Trauma is the leading cause of death and disability among young adults, which is often due to severe blood loss. Bleeding following trauma causes 1.5 million deaths a year worldwide.
South Africa has a high burden of trauma, especially amongst young adults.
Improvements in survival and better clinical outcomes from trauma result from early diagnosis, rapid bleeding control, and the early deployment of ambulances to rapidly transport patients to advanced medical care.

Reprinted from https://www.dvidshub.net/news/printable/311421 accessed 1/31/2021

Development of Third Generation HBOCs

After the setbacks experienced by the researchers of earlier generation HBOCs, manufacturers set out to develop products that would eliminate the toxicities, biophysical and chemical adverse events caused by the crosslinked/polymerized HBOCs. In lieu of creating products to replace donor blood for transfusions, researchers shifted focus to development of artificial oxygen carriers designed to be administered when erythrocytes are unavailable or in cases where a blood transfusion is impossible because of health or religious reasons (e.g., Jehovah's Witness). Researchers concluded that a blood substitute as an artificial oxygen carrier may be designed to prevent or treat ischemia-related morbidity and consequently decrease the mortality from anemia, hypoperfusion, or ischemia [7].

A product focused on alleviating ischemia related morbidities would establish enough effectiveness to gain regulatory approval, rather than a product designed to replace donor blood. Hemospan (MP4) was produced by Sangart in San Diego, CA, Hemospan was manufactured from outdated human blood and modified with maleimide-polyethylene glycol. Related to proposed non-hypertensive action, Hemospan was regarded as a plasma expander, in sharp distinction from previous vasoconstrictive HBOCs. MP4 initially had favorable results in its Phase I study, where healthy subjects did not become hypertension or experience gastrointestinal adverse effects [47].

While the MP4 itself was showing promise, further studies on it within the United States were halted. In 2008, Natanson et al. published a meta-analysis concerning the risk of myocardial infarction (MI) and death in patients participating in HBOC trials [48]. The meta-analysis itself was inconclusive and exhibited major statistical analysis incongruencies. Despite the study's methodological inconsistencies, the FDA implemented an immediate halt to all HBOC studies [49]. Fortunately, the regulatory agencies in the European Union and many other countries allowed HBOC clinical trials to continue once they had completed their own safety review.

Following trials testing Hemospan in trauma settings across various countries worldwide, the FDA requested more information to approve a Phase 2c. Although the FDA eventually allowed the trial, Sangart could not obtain enough funding to continue with the trial [49].

Another HBOC product focused on alleviating hypoxia and relieving ischemic tissue is Sanguinate, produced by Prolong Pharmaceuticals. Sanguinate is PEGylated bovine carboxyhemoglobin, which releases carbon monoxide (CO) and transfers O_2. However, this cannot be done simultane-

ously as they both bind the same binding site. It is suspected that they initially make carbonmonoxy-Sanguinate, and once infused, it is purported to release CO during circulation and subsequently it oxygenated (oxy- Sangunate) in the lung and delivers oxygen to the tissues. But the manufacturer never explicitly demonstrated data that explained how Sanguinate actually works.

This dual action was hypothesized to help inhibit vasoconstriction, diminish extravasation, decrease free oxygen radial formation, enhance blood rheology, and oxygenate tissues; however, these claims were not independently validated [50]. The dual action component, the purported safety profile and mechanism of action, sets it apart from other HBOCs, yet the manufacturer also attempted to develop and test CO-MP4.

Misra et al. led a Phase I clinical study, a single-center, single-blind, placebo-controlled, single dose study of the safety of Sanguinate in a group of healthy patients. The study indicated a good safety profile when administered at 80, 120, or 160 mg/kg.

Once its safety was established in healthy patients, Sanguinate was administered under an expanded access emergency Investigational New Drug (IND). The patient had sickle trait as well as beta thalassemia trait, and due to being a Jehovah's Witness, refused blood transfusions. The patient had a brain oximetry reading between the 40 and 50 mmHg range and was no longer responsive. Following administration to the patient brain oximetry values rose to normal range, patient regained mental status, and became responsive. The patient's improvement indicated Sanguinate had transported oxygen to the ischemic cerebral tissue [50].

In a randomized, open label, Phase Ib open label safety study of Sanguinate in sickle cell subjects, researchers found Sanguinate to be safe, with no serious treatment-related adverse events [51]. This study supports the concept that Sanguinate may be beneficial for the management of comorbidities associated with sickle cell anemia.

Another area in which Sanguinate might be beneficial is in the treatment of subarachnoid hemorrhage (SAH). Dhar et al. conducted a study evaluating administration of Sanguinate to patients with SAH at risk for delayed cerebral ischemia (DCI). The study was small, with only 12 patients. However, Sanguinate administration improved cerebral blood flow (CBF) and flow metabolism balance in the weak brain regions [52].

A newer HBOC product, Oxyvita is showing promise by eliminating the issues presented by earlier HBOC generations, however has not yet been tested clinically. Oxyvita is a zero-linked hemoglobin polymer, created by using cross-linked bovine tetramers instead of any exogenous binding agents previously designed in the manufacture of HBOCs, such as raffinose or glutaraldehyde. Without those exogenous binding agents remaining in the product and the supra-high molecular weight (20–30 million Dalton), Oxyvita has a much lower tendency to extravasate into the subendothelial space where NO scavenging may readily occur. Therefore, Oxyvita is purported to avoid NO scavenging, which was a common problem in previous HBOCs. Oxyvita can be stored safely at room temperature or stored in lyophilized form for up to 5 years. In its liquid form, it can be stored for 12 months at room temperature [27]. Its storing capabilities are a major advantage. In studies, Oxyvita has eliminated extravasation associated with HBOCs and shown to deliver oxygen to ischemic tissues more effectively than red blood cells [53]. Future studies are needed but so far, the product appears promising.

OxyVita is an HBOC that is developed to be different from others in that it ostensibly avoids vasoconstriction, hypothesized to be related to nitric oxide induction locally due to HBOC extravasating from the circulatory system (including lymph). OxyVita was not found in lymphatic fluid after an infusion in a feline model, nor did it demonstrate any vasoconstriction. Possibly, decreased extravasation thereby minimized the effects of nitric oxide being scavenged, therefore not causing the expected vasocontrictive issues.

The final HBOC product to be discussed is HemO2Life. HemO2Life is derived from extracellular hemoglobin (M101) from *Arenicola marina*, an invertebrate marine worm that experiences periods of hypoxia and hypothermia during high tide [14]. The heavy (3600 kDa) biopolymer exhibits a large O_2 binding capacity and releases O_2 without the assistance of allosteric effectors, via a simple gradient [54]. It has been marketed as an organ preservative to be added to solutions used for donated organs during transport.

It is being studied for organ preservation. A common complication in organ transplantation is the high chance of ischemia-reperfusion injuries [55]. There are procedures aimed at reducing enzymatic activity, and thus reducing the risk of ischemia, but the need for an efficient oxygen delivery system remains. Researchers wanted to examine the beneficial effects of $HemO_2$Life for kidney preservation prior to transplantation. Bovine kidneys were subjected to 60-minutes warm ischemia prior to preservation to increase post-transplant complications, in order to mimic kidney conditions witnessed in extended donor and deceased after circulatory death kidneys. The addition of $HemO_2$Life to the preservation solution decreased short-term function loss and preserved tissue integrity, overall improving organ preservation [55]. Following the success in bovine studies, $HemO_2$Life was tested for the first time in humans.

A multicenter safety study was conducted to study the addition of $HemO_2$Life to the preservative process of 60 kidneys harvested from deceased donors. The study conducted by Le Meur et al. indicated the transplanted kidneys from the $HemO_2$Life added solution presented less delayed graft function than the contralateral group of kidneys which were kept

in the preservation solution not containing $HemO_2Life$, in addition to presenting better renal function as measured by creatinine levels [56]. Preliminary data from completed trials looks favorable, with no adverse events, graft loss related to product, no deaths, and no allergic or immunological effects from $HemO_2Life$.

Coagulation and HBOCs

Introduction

Coagulation is a complex process that works by creating a chemical reaction, eliminating negative charges that cause particles to repel each other. The action of these bubbles forces clots or flocs of particles to the water surface where they can be skimmed off. Understanding the importance of coagulation, different scientists have developed different methods and coagulants in order to have the right procoagulants for the necessary indication [57]. However, whenever biologic therapeutics, such as HBOCs are developed, it is critical to determine if they are procoagulant or anticoagulant, as their effect to improve oxygen carrying capacity may be diminished or negated by clot formation or anticoagulant effects.

One initial concern with HBOC was possible activation of platelets as Hb can scavenge endothelial NO which has an anti-PLT aggregation effect. Therefore, with the development of Oxyvita, its possible coagulation side-effects, a new generation HBOC or Oxyglobin (HBOC-200, the veterinary congener to Hemopure) were studied [58]. This research aimed to describe and analyze the effects of Oxyvita or Oxyglobin and compared these effects with other hemodiluants and investigated if molecular weight and molecule specificity affects the results. Comparisons were made in low, medium, high and very high concentrations, using the measurements made with thrombelastography (TEG) [58].

TEG analysis demonstrated that OxyVita and Oxyglobin have the greatest deviation on clot strength. The size of this activity is equivalent to 6% hetastarch at low and medium dilutions, yet was significantly larger that that of 0.9% normal saline solution. At the highest dilutions, both OxyVita and Oxyglobin clot strength was decreased significantly in comparison to 6% hetastarch, 0.9% normal saline, and whole blood [58].

Oxidized Oxyglobin cause elevated methemoglobin levels may implicate that additional coagulation issues which are resulting most likely, by platelet function and coagulation proteins reaction to the effects of oxygen free radicals. These may interact with coagulation factors cause modification of the redox-sensitive areas on the platelet surface. It is entirely possible that methemoglobinemia, related to HBOC infusion, may cause levels to be greater than 10%, considered significant, that there may be coagulopathies as a result [59].

OxyVita is now also produced in a powder form, basically lyophilized from the liquid form [60]. This has been tempered with the need for proper buffering, ensuring appropriate electrolytes are maintained in the delivered product, and that the osmolarity is maintained in the final infusion solution. Reconstitution time may also be a factor in military and civilian trauma use [60].

Ex vivo comparisons of coagulation interference of two different molecular weight HBOCs (OxyVita, a new-generation zero-linked polymerized bovine hemoglobin-based oxygen carrier, 17–33 megadalton and Oxyglobin, 200 kilodalton) and hetastarch 6% (670 kilodalton) with normal saline, using thrombelastography, showed that the two HBOCs decreased clot strength (MA and G) at low and medium dilutions [61].

Since Oxyvita utilizes a modification of the zero-link polymerization, which uses chemical activators to add the inter-dimerically bovine hemoglobin that is cross-linked into "super-polymeric" macromolecules [62]. This unique design may also be of value with regard to coagulation concerns as demonstrated in the study previously discussed.

The new OxyVita that is lyophilized has properties similarly to the original liquid form after its reconstitution in an aqueous format. The time to reconstitute, based on solubility, with a time range of 10–30 s in water and has been demonstrated to function similarly to the liquid format [63].

Small volume resuscitation is now the recommended strategy for hemorrhaging patients; since most fluids currently advocated are larger volume with lack of oxygen carrying opportunities, HBOCs may be the idea solution to aid with oxygen deliver to deprived tissues [64].

This thinking then may be applied to the use of Hemopure in trauma victims: since it may not replace erythrocytes, its value to allow severely compromised victims to be rescued in the place of injury or in more advanced care settings, when either hematinic effects may allow for regeneration of erythrocytes, or transfusion is an option [65].

As a new generation HBOC, zero-linked hemoglobin polymer may begin to address the issues presented by the first two earlier generations of HBOCs, and may hold promise as a universally applicable HBOC [27].

The requirement for an alternative to erythrocytes to transport oxygen and moderate transfusions has driven the search for artificial oxygen carriers, mostly in the HBOC realm, as products derived from human or bovine Hb, or chemical modification or genetic engineering [66].

Finally, a citation of the Oxyvita study commented that the study was successful in mimicking resuscitation of traumatic hemorrhagic shock with the resultant hemodilution of crystalloids or colloid solutions [67]. Effects on coagulation when whole blood is diluted to 1:11, 1:5, 1:2, or 1:1 with

normal saline, 6% hetastarch(670 KDa), Hb-200, or OxyVita were analyzed accurately, and the results were considered valid and reproducible [67].

Single Site Clinical Study of Hemopure on Platelet Function

A single-site analysis from the previous HEM-0115 trial assessed platelet function in patients, before and after transfusion of erythrocytes or infusion of Hemopure [61]. The analysis using a PFA-100 (Platelet Function Analyzer-100, Siemens Healthcare Diagnostics Inc., Tarrytown, NY). "The PFA-100 System measures the complex process of primary haemostasis and aids in the rapid detection of platelet dysfunction. It is the first commercially available *in vitro* testing system to incorporate high-shear flow in which the process of platelet adhesion and aggregation following a vascular injury is simulated *in-vitro*" [68]. From the databank and unpublished, it was determined that those using Hemopure did not require more non-red blood cell products, such as fresh frozen plasma, cryoprecipitate and platelets, than the erythrocyte group (published as AABB Abstract: Williams J et al. Transfusion 2002). There was also a significant difference found in the "after transfusion" time period between the Hemopure and erythrocyte group, where the Hemopure group had increased cEPI and cADP (measurements of platelet adhesion that are aspirin-independent and aspirin-dependent). An increase in these measurements can correlate with an increased risk for bleeding. This increase, however, reversed about one Hemopure half-life on "Day 1 After transfusion." The increased cEPI may be explained by the lower level of Hb concentration found in the study in patients given Hemopure *versus* erythrocytes. Possibly, the increase in cEPI and cADP was due to haemodilution of the Hemopure blood product, and not the Hemopure itself. No change that was statistically significant in cEPI or cADP measurements from "before transfusion" and baseline was noted [61].

In this clinical trial, Hemopure's effects on subjects that undergo orthopaedic surgical interventions were evaluated. The research compared both packed red blood cells *versus* Hemopure at multiple periods [61], true baseline (before surgical incision and induction of anaesthesia), and at the following time periods: prior to the decision to transfuse, following an episode of transfusion, on the first, second, and third through ninth days and at follow-up of 21 days following a transfusion episode. In this subset study, 27 subjects were evaluated, 12 in the Hemopure group and 15 in PRBC.

Prior to transfusion and at baseline comparisons did not reveal a significant changes statistically in any of cEPI-collagen/epinephrine or cADP-collagen/ADP evaluations. cEPI results of the Hemopure cohort elevated (not statistically significant) compared with the true baseline, after transfusion, before transfusion and a day after transfusion. cADP results in the Hemopure cohort were larger post-transfusion in comparison to the actual baseline and before transfusion. At all intervals, no differences that were statistically valid were noted in the PRBC group in cEPI and cADP results.

The trial revealed that Hemopure infusion appears to create some platelet aggregation issue. Despite statistically significant differences after Hemopure infusion, the mean values of cEPI and cADP were greater than the documented upper normal limits, the values were not above the non-closure time, indicating that clotting could still occur.

In an animal study for the effects of Hemopure on platelet function, it was demonstrated that animals that were resuscitated with Hemopure or hetastarch 6% both, initially, had significant increases in the platelet function analyser closure time (PFA-CT). This change returned to normal in the hetastarch group by 24 hours but reached a maximum in the HBOC-201 group at 24 hours and normalised in 72 hours after transfusion [69]. This increase in both Hemopure and hetastarch is likely due to the glycoproteins that are on the surface of both these molecules that have transient effects on platelet function, yet do not destroy function such that the platelets are functionless, as with chronic aspirin therapy.

Ex vivo comparisons of coagulation interference of two different molecular weight HBOCs and hetastarch 6% with normal saline, using thrombelastography, showed that the two HBOCs decreased clot strength (MA and G) when evaluated at dilutions considered to be low and medium. The effect's noted size was similar to 6% hetastarch but was larger significantly than a normal saline solution would be expected to produce [58]. This *ex-vivo* study sheds important light on the platelet function study in that many subjects in the HEM-0115 trial were treated with 6% hetastarch as a volume expander and it has definite effects on platelet function as demonstrated in this TEG study.

The analysis using a PFA-100 determined that those using Hemopure did not require more blood products than the erythrocyte group. There was also a significant difference found in the "after transfusion" time period between the Hemopure and erythrocyte groups, where the HBOC-201 group had increased cEPI and cADP. An increase in these measurements can correlate with an increased risk for bleeding [70]. This increase, however, reversed about one Hemopure half-life on "Day 1 After transfusion."

The increased cEPI may be explained by the lower level of Hb concentration found in the study in patients given Hemopure *versus* erythrocytes. It is also possible that the increase in cEPI and cADP was due to haemodilution of the Hemopure blood product, and not the Hemopure itself. There was no statistically significant change in cEPI or cADP measurements from "before transfusion" and baseline.

How do cEPI and cADP values change in different diseases and conditions according to this work? In PFA-100 measurements, the cEPI cartridge is more sensitive overall

(86%) than the cADP cartridge (81%) and is especially sensitive to drug-related platelet dysfunction [70]. The clinical predictive value of PFA-100 is debatable, because severe bleeding caused by thrombocytopenia is rare and occurs only in patients with a concomitant coagulopathy or anatomical defects in the vascular system [71].

The effects on platelet function that are made by anaesthetic inhalational agents such as halothane, sevoflurane, and intravenous anesthetic agents such as propofol cause a reversible inhibition of platelet function, which is dose dependent in doses utilized in clinical practice. There is a residual suppressive effect 1 hour postoperatively with sevoflurane and propofol [72]. Although the research study has shown that by comparing the baseline values with before transfusion time period, the anaesthetic drugs or surgery has no significant effect on PFA-CT. Certain food molecules and foods are known to inhibit platelet function as well such as fatty acids and cocoa [72].

Cell-free Hb is prone to auto-oxidation to methaemoglobin; Hemopure infusion in surgical patients demonstrated that the percent of plasma methaemoglobin increased in a dose-dependent manner with a delayed onset and reached maximal value 3 days after transfusion [73]. The mean value of methaemoglobin concentration was 3.66% and, in patients who received high doses of Hemopure (2.5 g/kg), the mean was 7.1%. In the process of oxidation, reactive oxygen species are generated. Platelets contain several glycoprotein receptors with thiol groups and vicinal thiols, making them redox-sensitive structures. These glycoprotein receptors include adhesion receptor glycoprotein IIb/IIIa and the P2Y12ABT ADP receptor, which is instrumental in the function of platelet's activation and aggregation. Disulphide isomerase such as protein disulphide isomerase, which has a role in platelet aggregation, is another redox-sensitive structure. A redox homeostasis exists in blood as a result of a trans-plasma membrane redox system of platelets, which can be impaired by free radicals [73].

This recent supportive citation of the earlier work helps understand how these platelet function changes might be explained.

Hemopure has a major impact as an HBOC in research on the human liver based on a study that used Hemopure in a novel indication, where oxygen supply is needed and prior to an HBOC availability, only erythrocytes could deliver such.

Investigators published the first use of Hemopure in a model of liver perfusion requiring machine perfusion (NMP-L). This study suggests that Hemopure compared to erythrocytes, may provide the following advantages: in terms of logistics, rheology, and immunology, in the evaluation of a perfusion model using normothermia [74].

In dealing with myocardial infarction (MI) and its incidence with HBOC studies based on investigator diagnosis, imbalances have been noted and possible toxicity mechanisms have been proposed to help understand these differences [75]. Experiments have been conducted that evaluated in vitro combining of HBOCs (made with human haemoglobin, unlike Hemopure, which uses bovine) and whole blood, the mixture may not cause aggregation of platelets or their activation, nonetheless, certain agonists' responses may be augmented [76]. However, HBOCs with a bovine haemoglobin have been suggested to cause clot formation impairment [75]. This important review by one of the investigators in a first generation HBOC, is important and supportive, in that if HBOCs cause coagulopathies, then it may diminish their ability to help resuscitate a trauma victim or other patient with massive blood loss and acute anaemia.

Coagulation is largely dependent on adequate platelet activity. High molecular weight polymers present in hetastarch solutions have been demonstrated to be related to coagulopathy. In this study, Hemopure was compared to erythrocytes. Mild platelet dysfunction was documented [77]. This review also reconfirms the statements from Reference [76].

Donated human blood is the source of all erythrocyte and plasma products, and so its viral safety is crucial Currently, lipid-enveloped viruses, such as human immunodeficiency virus, hepatitis C virus and hepatitis B virus risks from donated blood products is almost non-existent with appropriate testing in blood banks [78]. While the comments about the incidence of viral contaminants to the blood supply in the past have been widely dealt with, the newer viruses may always cause issues (COVID-19) and the supply of donors is shrinking as populations age and demand increases. Therefore, it may be short-sighted to dismiss the need for these types of products, notwithstanding the inherent risks of transfusion.

Summary of Hemopure

Hemopure represents the most widely studied commercially developed polymerized Hbs. The chemical cross-links within these HBOCs are random and occur both intermolecularly and intramolecularly. The HBOCs generated as a result are large molecules and were hypothesized to negate the previously mentioned side-effects associated with transfusion of acellular HBOCs, such as, vasoconstriction and hypertension engendered by the remants of cell-free Hb. Unfortunately, clinical studies conducted with both of these polymerized Hbs elicited substantial hypertension in vivo upon transfusion [79].

Since erythrocytes and all other blood products come from donated human blood, safety of viral contamination is paramount, not so much for the risk of transmission of lipid-enveloped viruses, such as HIV, Hepatitis C, and Hepatitis B, the transmission by plasma-derived solutions is likely minimal [78]. However, there are new viruses constantly being identi-

fied, such as COVID, which does not seem to be transmitted in donated blood, although the detection and removal processes of blood banks take years to initiate and implement.

Summary and Moving Forward

This chapter has reviewed HBOCs from early until the most recently completed trials, as well as discussed how various HBOCs are made and their pharmacology and physiology. Donated human blood and the derived products that have been available for around 50 years have allowed for replacement of erythrocytes, platelets and blood clotting factors and plasma/albumin. Based on the available published articles on products and in some cases recent website reviews, this chapter has updated the reader on the current usage of some products and attempted to focus on the risk/benefit of current products and attempted to introduce the reader to newer products that may someday improve outcomes from situations where blood is not available or an option [70, 80–83].

Donor erythrocyte transfusion may be a lifesaving treatment for severe anemic conditions. However, donor blood is a precious commodity that is in chronic shortage because there is no alternative. Aging populations and changes in the population dynamics worldwide will further worsen blood shortages. In the coming decades, it is predicted that the number of older people who will need blood transfusion will far exceed the younger people who can donate blood. Without alternatives, this imbalance may cause a blood shortage crisis that could result in countless preventable deaths. To alleviate the current shortages and prevent future blood supply crisis, we must invest now to reinvigorate efforts to develop of safe and effective blood substitute(s).

Since the early 1980's, HBOCs based on modified (cross-linked, polymerized or conjugated) human or bovine Hbs have been developed as leading candidates for donor RBC substitutes and some have recently been tested in clinical trials (HemAssist, Hemopure, PolyHeme, Hemolink, Hemospan, Hemoximer, Sanguinate). However, in phase 2–3 clinical trials, some adverse events/serious adverse events occurred in the HBOC treated subjects including pulmonary and systemic hypertension, cardiac dysfunction, coagulopathy and enzyme abnormalities [76]. This engendered the early cessation of studies or completed Phase 3 trial but failure to obtain regulatory clearance due to safety concerns.

Although Hemopure secured regulatory approval for human use in anemia in South Africa and Russia, none of the HBOC products tested have been approved for clinical use in the US and other advanced economies, except for Oxyglobin, the veterinary product approved by the United State Food and Drug Administration and its counterparts in the European Union and United Kingdom.

It is generally agreed that the pulmonary and systemic hypertensive responses observed following HBOC administration were caused by the iron-heme, a common prosthetic group in all the HBOCs, which avidly scavenges the vascular endothelium derived NO, a potent vasodilator, causing vasoconstriction leading to hypertension. NO has also been discovered to be produced endogenously in the cardiomyocytes to contribute significantly in the regulation of cardiac contractility and rhythm. Therefore, a possibility has been raised that HBOC interference with cardiac NO signaling might have caused some of the cardiac adverse events observed. In addition, HBOC redox-mediated radical generation and other unknown toxicity mechanism may also be involved. However, mechanisms for other adverse events/serious adverse events have not been elucidated.

Considering the issues encountered in HBOC clinical trials, a newer generation of HBOCs must be developed that significantly reduces NO reactivity and yet maintains optimal oxygen binding and delivery characteristics. In addition, it is highly desirable that newer HBOCs have favorable redox properties and stability for an extended storage preferably without refrigeration.

These are scientifically challenging goals but, with strong research support for better understanding of chemistry, physiology and pharmacology of HBOCs in clinically relevant models, they are achievable. Unfortunately, recent failures in obtaining regulatory approval and negative media coverage have caused a devastating impact on the HBOC field. However, there are a number of promising new products being developed and tested, many at the most basic level either in university or manufacturer settings.

Despite the critical need and urgency, current negative environment and lack of government support, make it extremely difficult to secure necessary funding for new HBOC development. Under the current circumstances and limited resources, a collaborative and coordinated approach among academia (basic research with funds from government and industry), industry (scaleup and manufacturing with angel/venture capital funding) and government (provide startup funding and regulatory support) would help create development of new or improved HBOCs for successful regulatory approval and eventual clinical use [84]. With an initial government funding, such a consortium could be implemented and start operationalized with an aim to help facilitate development of one or more safe and effective blood substitutes in a decade or two. Additionally, currently tested HBOCs, such as Hemopure, and newer products like Hemarina's HEMO2-life may be safe enough to provide a life-savings advantage, despite the side effect profile, in those subsets of patients who are less affected by them and benefit from the value of improved oxygen delivery in the face of severe anemia or when blood is not available or an option.

Key Points

- Blood is a donated product which is soon to be in short supply due to older population, fewer donors and more demand
- Hemoglobin-based oxygen carriers have been developed to ameliorate this shortfall and deliver oxygen to hypoxic tissues for any etiology: acute anemia or hemorrhage
- Hemoglobin-based oxygen carriers have intrinsic side effects, like all drugs, which must be understood and circumvented to achieve the desired benefits
- Newer generation HBOCs have been formulated to avoid the side effects of NO scavenging, hypertension, and chemical pancreatitis, or products to give as adjuncts
- Hemopure is approved for human use in South Africa and Russia and available for Expanded Use; Oxyglobin is approved for veterinary use in the US and EU
- Hemarina, made from sea worm's hemoglobin that can survive in air at low tide, may provide oxygenation from a natural and unaltered hemoglobin that is naturally able to provide more oxygen due to its biomimicry

References

1. Jahr JS, Walker V, Manoochehri K. Blood substitutes as pharmacotherapies in clinical practice. Curr Opin Anaesthesiol. 2007;20(4):325–30.
2. Schubert A, Przybelski RJ, Eidt JF, Lasky LC, Marks KE, Karafa M, et al. Diaspirin-crosslinked hemoglobin reduces blood transfusion in noncardiac surgery: a multicenter, randomized, controlled, double-blinded trial. Anesth Analg. 2003;97(2):323–32, table of contents.
3. Kasper SM, Walter M, Grüne F, Bischoff A, Erasmi H, Buzello W. Effects of a hemoglobin-based oxygen carrier (HBOC-201) on hemodynamics and oxygen transport in patients undergoing preoperative hemodilution for elective abdominal aortic surgery. Anesth Analg. 1996;83(5):921–7.
4. Greenburg AG, Kim HW. Use of an oxygen therapeutic as an adjunct to intraoperative autologous donation to reduce transfusion requirements in patients undergoing coronary artery bypass graft surgery. J Am Coll Surg. 2004;198(3):373–83; discussion 84–5.
5. Levy JH, Goodnough LT, Greilich PE, Parr GV, Stewart RW, Gratz I, et al. Polymerized bovine hemoglobin solution as a replacement for allogeneic red blood cell transfusion after cardiac surgery: results of a randomized, double-blind trial. J Thorac Cardiovasc Surg. 2002;124(1):35–42.
6. Rentko V, Pearce B, Moon-Massat P, Gawryl M. Hemopure® (HBOC-201, Hemoglobin Glutamer-250 (Bovine)): preclinical studies. Blood Substitutes. Burlington, MA: Academic Press; 2006. p. 424–36.
7. Khan F, Singh K, Friedman MT. Artificial blood: the history and current perspectives of blood substitutes. Discoveries (Craiova). 2020;8(1):e104.
8. Roghani K, Holtby RJ, Jahr JS. Effects of hemoglobin-based oxygen carriers on blood coagulation. J Funct Biomater. 2014;5(4):288–95.
9. Rosenblatt MA. Strategies for minimizing the use of allogeneic blood during orthopedic surgery. Mt Sinai J Med. 2002;69(1–2):83–7.
10. Habler O, Meier J, Pape A, Zwissler B. Indications for blood transfusion during orthopedic surgery. Orthopade. 2004;33(7):774–83.
11. Goodnough LT, Brecher ME, Kanter MH, AuBuchon JP. Transfusion medicine. First of two parts – blood transfusion. N Engl J Med. 1999;340(6):438–47.
12. Vamvakas EC. Epidemiology of red blood cell utilization. Transfus Med Rev. 1996;10(1):44–61.
13. Tobias JD. Strategies for minimizing blood loss in orthopedic surgery. Semin Hematol. 2004;41(1 Suppl 1):145–56.
14. Jahr JS, Guinn NR, Lowery DR, Shore-Lesserson L, Shander A. Blood substitutes and oxygen therapeutics: a review. Anesth Analg. 2021;132(1):119–29.
15. De Venuto F, Zuck TF, Zegna AI, Moores WY. Characteristics of stroma-free hemoglobin prepared by crystallization. J Lab Clin Med. 1977;89(3):509–16.
16. Winslow RM, Vandegriff KD. Inventors method for production of stroma-free hemoglobin. United States of America 2004. https://patents.google.com/patent/US7989414B2/en. Accessed 03/01/2021.
17. Cheung LC, Storm CB, Gabriel BW, Anderson WA. The preparation of stroma-free hemoglobin by selective DEAE-cellulose absorption. Anal Biochem. 1984;137(2):481–4.
18. Snyder SR, Welty EV, Walder RY, Williams LA, Walder JA. HbXL99 alpha: a hemoglobin derivative that is cross-linked between the alpha subunits is useful as a blood substitute. Proc Natl Acad Sci. 1987;84(20):7280–4.
19. Walder JA, inventor; University of Iowa Research Foundation (Iowa City, IA, US), assignee. Production of alpha-alpha cross-linked hemoglobins in high yield. United States patent US Patent Re. 34,271. 1993.
20. Chevalier A, Guillochon D, Nedjar N, Piot JM, Vijayalakshmi MW, Thomas D. Glutaraldehyde effect on hemoglobin: evidence for an ion environment modification based on electron paramagnetic resonance and Mossbauer spectroscopies. Biochem Cell Biol. 1990;68(4):813–8.
21. Varnado CL, Mollan TL, Birukou I, Smith BJZ, Henderson DP, Olson JS. Development of recombinant hemoglobin-based oxygen carriers. Antioxid Redox Signal. 2013;18(17):2314–28.
22. Natarajan C, Jiang X, Fago A, Weber RE, Moriyama H, Storz JF. Expression and purification of recombinant hemoglobin in Escherichia coli. PLoS One. 2011;6(5):e20176.
23. Bucci E, Kwansa H, Koehler RC, Matheson B. Development of zero-link polymers of hemoglobin, which do not extravasate and do not induce pressure increases upon infusion. Artif Cells Blood Substit Immobil Biotechnol. 2007;35(1):11–8.
24. Ea A, Ronda L, Bruno S, Paredi G, Marchetti M, Bettati S, et al. High- and low-affinity PEGylated hemoglobin-based oxygen carriers: differential oxidative stress in a Guinea pig transfusion model. Free Radic Biol Med. 2018;124:299–310.
25. Alix P, Val-Laillet D, Turlin B, Ben Mosbah I, Burel A, Bobillier E, et al. Adding the oxygen carrier M101 to a cold-storage solution could be an alternative to HOPE for liver graft preservation. JHEP Rep. 2020;2(4):100119.
26. Lupon E, Lellouch AG, Zal F, Cetrulo CL Jr, Lantieri LA. Combating hypoxemia in COVID-19 patients with a natural oxygen carrier, HEMO(2)Life® (M101). Med Hypotheses. 2021;146:110421.
27. Jahr JS, Akha AS, Holtby RJ. Crosslinked, polymerized, and PEG-conjugated hemoglobin-based oxygen carriers: clinical safety and efficacy of recent and current products. Curr Drug Discov Technol. 2012;9(3):158–65.
28. Winslow RM. Alphaalpha-crosslinked hemoglobin: was failure predicted by preclinical testing? Vox Sang. 2000;79(1):1–20.
29. Hess JR. Review of modified hemoglobin research at letterman: attempts to delineate the toxicity of cell-free tetrameric hemoglobin. Artif Cells Blood Substit Immobil Biotechnol. 1995;23(3):277–89.

30. Saxena R, Wijnhoud AD, Carton H, Hacke W, Kaste M, Przybelski RJ, et al. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke. Stroke. 1999;30(5):993–6.
31. Sloan EP, Koenigsberg M, Gens D, Cipolle M, Runge J, Mallory MN, et al. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial. JAMA. 1999;282(19):1857–64.
32. Cheng DC, Mazer CD, Martineau R, Ralph-Edwards A, Karski J, Robblee J, et al. A phase II dose-response study of hemoglobin raffimer (Hemolink) in elective coronary artery bypass surgery. J Thorac Cardiovasc Surg. 2004;127(1):79–86.
33. Hill SE, Gottschalk LI, Grichnik K. Safety and preliminary efficacy of hemoglobin raffimer for patients undergoing coronary artery bypass surgery. J Cardiothorac Vasc Anesth. 2002;16(6):695–702.
34. Greenburg AG, Kim HW. Hemoglobin-based oxygen carriers. Crit Care. 2004;8 Suppl 2(Suppl 2):S61–4.
35. Salazar Vázquez BY, Wettstein R, Cabrales P, Tsai AG, Intaglietta M. Microvascular experimental evidence on the relative significance of restoring oxygen carrying capacity vs. blood viscosity in shock resuscitation. Biochim Biophys Acta. 2008;1784(10):1421–7.
36. Moore EE, Moore FA, Fabian TC, Bernard AC, Fulda GJ, Hoyt DB, et al. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. J Am Coll Surg. 2009;208(1):1–13.
37. Jahr JS, Mackenzie C, Pearce LB, Pitman A, Greenburg AG. HBOC-201 as an alternative to blood transfusion: efficacy and safety evaluation in a multicenter phase III trial in elective orthopedic surgery. J Trauma. 2008;64(6):1484–97.
38. Practice guidelines for perioperative blood transfusion and adjuvant therapies: an updated report by the American Society of Anesthesiologists Task Force on Perioperative Blood Transfusion and Adjuvant Therapies. Anesthesiology. 2006;105(1):198–208.
39. McClelland DBL, editor. Handbook of transfusion medicine. 4th ed. London, UK: United Kingdom Blood Services; 2007.
40. Murphy MF, Wallington TB, Kelsey P, Boulton F, Bruce M, Cohen H, et al. Guidelines for the clinical use of red cell transfusions. Br J Haematol. 2001;113(1):24–31.
41. Practice Guidelines for blood component therapy. A report by the American Society of Anesthesiologists Task Force on blood component therapy. Anesthesiology. 1996;84(3):732–47.
42. Go AS, Yang J, Ackerson LM, Lepper K, Robbins S, Massie BM, et al. Hemoglobin level, chronic kidney disease, and the risks of death and hospitalization in adults with chronic heart failure: the Anemia in Chronic Heart Failure: Outcomes and Resource Utilization (ANCHOR) Study. Circulation. 2006;113(23):2713–23.
43. Kim HW, Tai J, Greenburg AG. Active myogenic tone: a requisite for hemoglobin mediated vascular contraction? Artif Cells Blood Substit Immobil Biotechnol. 2004;32(3):339–51.
44. Kim HW, Greenburg AG. Mechanisms for vasoconstriction and decreased blood flow following intravenous administration of cell-free native hemoglobin solutions. Adv Exp Med Biol. 2005;566:397–401.
45. Chen JY, Scerbo M, Kramer G. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. Clinics (Sao Paulo). 2009;64(8):803–13.
46. LaMuraglia GM, O'Hara PJ, Baker WH, Naslund TC, Norris EJ, Li J, et al. The reduction of the allogenic transfusion requirement in aortic surgery with a hemoglobin-based solution. J Vasc Surg. 2000;31(2):299–308.
47. Björkholm M, Fagrell B, Przybelski R, Winslow N, Young M, Winslow RM. A phase I single blind clinical trial of a new oxygen transport agent (MP4), human hemoglobin modified with maleimide-activated polyethylene glycol. Haematologica. 2005;90(4):505–15.
48. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA. 2008;299(19):2304–12.
49. Keipert PE. Hemoglobin-based oxygen carrier (HBOC) development in trauma: previous regulatory challenges, lessons learned, and a path forward. Adv Exp Med Biol. 2017;977:343–50.
50. Misra H, Lickliter J, Kazo F, Abuchowski A. PEGylated carboxyhemoglobin bovine (SANGUINATE): results of a phase I clinical trial. Artif Organs. 2014;38(8):702–7.
51. Misra H, Bainbridge J, Berryman J, Abuchowski A, Galvez KM, Uribe LF, et al. A phase Ib open label, randomized, safety study of SANGUINATE™ in patients with sickle cell anemia. Rev Bras Hematol Hemoter. 2017;39(1):20–7.
52. Dhar R, Misra H, Diringer MN. SANGUINATE™ (PEGylated carboxyhemoglobin bovine) improves cerebral blood flow to vulnerable brain regions at risk of delayed cerebral ischemia after subarachnoid hemorrhage. Neurocrit Care. 2017;27(3):341–9.
53. Cabrales P, Sun G, Zhou Y, Harris DR, Tsai AG, Intaglietta M, et al. Effects of the molecular mass of tense-state polymerized bovine hemoglobin on blood pressure and vasoconstriction. J Appl Physiol (1985). 2009;107(5):1548–58.
54. Kasil A, Giraud S, Couturier P, Amiri A, Danion J, Donatini G, et al. Individual and combined impact of oxygen and oxygen transporter supplementation during kidney machine preservation in a porcine preclinical kidney transplantation model. Int J Mol Sci. 2019;20(8):1992.
55. Kaminski J, Hannaert P, Kasil A, Thuillier R, Leize E, Delpy E, et al. Efficacy of the natural oxygen transporter HEMO(2) life(®) in cold preservation in a preclinical porcine model of donation after cardiac death. Transpl Int. 2019;32(9):985–96.
56. Le Meur Y, Badet L, Essig M, Thierry A, Büchler M, Drouin S, et al. First-in-human use of a marine oxygen carrier (M101) for organ preservation: a safety and proof-of-principle study. Am J Transplant. 2020;20(6):1729–38.
57. Palta S, Saroa R, Palta A. Overview of the coagulation system. Indian J Anaesth. 2014;58(5):515–23.
58. Jahr JS, Weeks DL, Desai P, Lim JC, Butch AW, Gunther R, et al. Does OxyVita, a new-generation hemoglobin-based oxygen carrier, or oxyglobin acutely interfere with coagulation compared with normal saline or 6% hetastarch? An ex vivo thromboelastography study. J Cardiothorac Vasc Anesth. 2008;22(1):34–9.
59. Moallempour M, Jahr JS, Lim JC, Weeks D, Butch A, Driessen B. Methemoglobin effects on coagulation: a dose-response study with HBOC-200 (Oxyglobin) in a thrombelastogram model. J Cardiothorac Vasc Anesth. 2009;23(1):41–7.
60. Harrington JP, Wollocko H. Molecular design properties of OxyVita hemoglobin, a new generation therapeutic oxygen carrier: a review. J Funct Biomater. 2011;2(4):414–24.
61. Jahr JS, Liu H, Albert OK, Gull A, Moallempour M, Lim J, et al. Does HBOC-201 (Hemopure) affect platelet function in orthopedic surgery: a single-site analysis from a multicenter study. Am J Ther. 2010;17(2):140–7.
62. Harrington J, Wollocko H. Zero-link hemoglobin (OxyVita®): impact of molecular design characteristics on pre-clinical studies. In: Hemoglobin-based oxygen carriers as red cell substitutes and oxygen therapeutics. Berlin, Heidelberg: Springer Berlin Heidelberg; 2014. p. 283–97.
63. Harrington JP, Wollocko J, Kostecki E, Wollocko H. Physicochemical characteristics of OxyVita hemoglobin, a zero-linked polymer: liquid and powder preparations. Artif Cells Blood Substit Immobil Biotechnol. 2011;39(1):12–8.
64. Reynolds PS, Barbee RW, Skaflen MD, Ward KR. Low-volume resuscitation cocktail extends survival after severe hemorrhagic shock. Shock. 2007;28(1):45–52.

65. Jahr JS, Moallempour M, Lim JC. HBOC-201, hemoglobin glutamer-250 (bovine), Hemopure (Biopure Corporation). Expert Opin Biol Ther. 2008;8(9):1425–33.
66. Kluger R, Foot JS, Vandersteen AA. Protein-protein coupling and its application to functional red cell substitutes. Chem Commun (Camb). 2010;46(8):1194–202.
67. Jahr J, Driessen B, Gunther R, Lurie F, Lin J, Cheung A. Translational advances in second and third generation hemoglobin based oxygen carriers: one research team's perspective. Artif Blood. 2009;17:1–7.
68. https://www.siemens-healthineers.com/en-us/hemostasis/systems/pfa-100. Accessed 16 Jan 2021.
69. Arnaud F, Hammett M, Asher L, Philbin N, Rice J, Dong F, et al. Effects of bovine polymerized hemoglobin on coagulation in controlled hemorrhagic shock in swine. Shock. 2005;24(2):145–52.
70. Chung M, Mayer L, Nourmand H, You M, Jahr J. Chapter 26: blood, blood products, and substitutes. In: Kaye A, Kaye A, Urman R, editors. Essentials of pharmacology for anesthesia, pain medicine, and critical care. New York: Springer; 2015. p. 421–32.
71. Kozek-Langenecker SA. The effects of drugs used in anaesthesia on platelet membrane receptors and on platelet function. Curr Drug Targets. 2002;3(3):247–58.
72. Jilma B. Platelet function analyzer (PFA-100): a tool to quantify congenital or acquired platelet dysfunction. J Lab Clin Med. 2001;138(3):152–63.
73. Essex DW, Li M. Redox modification of platelet glycoproteins. Curr Drug Targets. 2006;7(10):1233–41.
74. Laing RW, Bhogal RH, Wallace L, Boteon Y, Neil DAH, Smith A, et al. The use of an acellular oxygen carrier in a human liver model of normothermic machine perfusion. Transplantation. 2017;101(11):2746–56.
75. Estep TN. Haemoglobin-based oxygen carriers and myocardial infarction. Artif Cells Nanomed Biotechnol. 2019;47(1):593–601.
76. Silverman TA, Weiskopf RB. Hemoglobin-based oxygen carriers-current status and future directions. Anesthesiology. 2009;111:946–63.
77. Roghani K, Holtby RJ, Jahr JS. Effects of haemoglobin-based oxygen carriers on blood coagulation. J Funct Biomater. Columbus, OH. 2014;5(4):288–95. https://doi.org/10.3390/jfb5040288.
78. Velthove KJ, Strengers PFW. 33 - Blood, blood components, plasma, and plasma products. In: Aronson JK, editor. Side effects of drugs annual. 34. Elsevier; 2012. p. 509–29.
79. Rameez S. Engineering cellular hemoglobin-based oxygen carriers for use in transfusion medicine. The Ohio State University; 2011.
80. Jahr JS. Editorial: Do approved blood substitutes reduce myocardial infarction size: is this the critical question? Br J Anaesth. 2009;103:470–1.
81. Jahr JS. Editorial: are newer generation hemoglobin-based oxygen carriers safe: do they offer an answer? Vasc Pharmacol. 2011;52:214.
82. Jahr JS, Sadighi A, Doherty L, Kim HW. Hemoglobin-based oxygen carriers: history, limits, brief summary of the state of the art, including clinical trials. In: Bettati S, Mozzarelli A, editors. Oxygen therapeutics: from transfusion to artificial blood. New York: Wiley.
83. Kim HW, Mozzarelli A, Sakai H, Jahr JS. Academia–industry collaboration in blood substitute development: issues, case histories and a proposal. Chemistry and biochemistry of oxygen therapeutics. In: Kim HW, Greenburg AG, editors. Hemoglobin-based oxygen carriers as red cell substitutes and oxygen therapeutics. Berlin, Heidelberg: Springer Berlin Heidelberg; 2013. p. 413–28.
84. Kim HW, Jahr JS, Mozzarelli A, Sakai H. International consortium for development of hemoglobin-based oxygen carriers, oxygen therapeutics and multifunctional resuscitation fluids–a white paper. In: Kim HW, Greenburg AG, editors. Hemoglobin-based oxygen carriers as red cell substitutes and oxygen therapeutics. Berlin, Heidelberg: Springer Berlin Heidelberg; 2013. p. 737–46.

Complications of HBOCs Including Clinical Safety Issues

13

Ahmad Alli, Aleksander Dokollari, and C. David Mazer

Introduction

Hemoglobin-based oxygen carriers (HBOCs) have been developed to treat anemia because of limited availability of packed red blood cells and concerns about the safety of allogeneic blood [1]. Several products have been developed but the pathway to clinical use has been complicated by differences in the products, unsuccessful clinical trials, differences in interpretation of study results and potential safety issues. These range from mild effects such as transient liver enzyme increases to myocardial infarction (MI) and stroke; with the more serious adverse events being attributed to higher mortality and morbidity in patients who receive some of these compounds [2]. Vasoconstriction secondary to the nitric oxide scavenging action of free hemoglobin is postulated to be an underlying pathophysiology contributing to possible hypertension, reduced oxygen delivery and organ ischemia [3]. HBOC manufacturers have attempted to reduce these effects by molecular modifications, and each subsequent generation of HBOCs seems safer than the previous. Despite many candidate molecules having positive Phase I and even Phase II/III trials [4], only a handful of products remain available for clinical and research use today. Neutral or negative outcomes in specific groups of patients led regulatory authorities such as the FDA to halt progression into Phase III trials, and subsequently many companies abandoned further research or shut down. A controversial negative meta-analysis by Natanson et al. in 2008 [5] attracted widespread criticism and is thought to have significantly impacted further research and development into HBOCs.

A. Alli (✉)
St. Michael's Hospital, Department of Anesthesiology and Pain Medicine, University of Toronto, Toronto, ON, Canada
e-mail: ahmad.alli@unityhealth.to

A. Dokollari
CARIM, School for cardiovascular diseases, Department of Cardiac Surgery, University of Maastricht, Maastricht, The Netherlands

C. D. Mazer
Li Ka Shing Knowledge Institute of St Michael's Hospital, Departments of Anesthesiology Pain Medicine and Physiology, University of Toronto, Toronto, ON, Canada

Department of Anesthesia, Unity Health Toronto, Toronto, ON, Canada
e-mail: David.Mazer@unityhealth.to

Criticism of the Natanson et al. Meta-Analysis

Natanson et al. published a meta-analysis in 2008 analysing 16 clinical HBOC trials [5]. Five different products were included in the study. These were HemAssist (9 studies), Hemopure (1 data pool from an FDA presentation), Hemolink (2 studies), Polyheme (3 studies) and Hemospan (1 study). Press releases and FDA hearing data were included as study data, and trials with healthy volunteers were excluded. Study data encompassed elective surgery, trauma and stroke patients. Primarily, mortality and MI were identified as the outcomes of interest. It was suggested that patients receiving HBOCs had an increased relative risk of death (RR 1.30, 95% CI 1.05–1.61). In terms of MI, it was reported that patients receiving HBOCs also had a significantly increased risk (RR 2.71, 95% CI 1.67–4.40). Trauma patients did not have a significant increase in MI or mortality, and perhaps this reflects the relatively younger age of this sub-group. Less well explained by age is the non-significant difference in MI and mortality in cardiac surgery patients. The authors concluded that further clinical trials of HBOCs should cease until more pre-clinical animal data, particularly focused on toxicities, was published. They also found that there were delays of 3–5 years in the publication of negative trial outcomes by HBOC manufacturers, and that if such delays had not occurred, clinical trials could have been halted earlier, and deaths may have been prevented.

This meta-analysis has been widely criticized and several problems have been elucidated. Firstly, the analysis treats the various HBOC compounds as equivalent. As is clear in the

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_13

preceding content within this book, the molecular differences between HBOC compounds results in vastly different pharmacodynamic properties, particularly regarding nitric oxide interactions and oxygen binding affinity. Indeed, when the authors of the study divided the analysis into low tetramer and high tetramer groups, the risk of MI or mortality is not significantly different in the high tetramer grouping (with the confidence interval crossing 1). This implies that the newer generation, higher tetramer, molecules are safer. Unfortunately, this finding is not expanded upon in the discussion. Secondly, the included control groups were dissimilar, patients in the control groups received blood, crystalloid or colloids and these patients were erroneously combined in the pooled control group. As has been debated for the last two decades in the critical care literature, colloid and crystalloid have different effects on clinical outcomes [6]. Particularly, the control group was specific for the clinical context of the patient, for example blood is more appropriate for use in traumatic hemorrhage than in patients undergoing elective surgical blood loss. Thirdly, various dissimilar clinical situations were included: patients undergoing resuscitation for trauma or stroke were included in the same group as those presenting for elective surgery. Volume expansion and nitric oxide scavenging effects in shocked trauma patients may be beneficial, whereas these effects could potentially be harmful in euvolemic, normotensive elective surgical patients. Patients in these various clinical situations also differ in underlying risk factors, for example, patients undergoing vascular surgery are at higher risk of myocardial infarction and death. Lastly, doses of product ranged from 50-3000 mL and dosing periods varied from 1 to 6 days. From a statistical point of view, a fixed effects model was used to pool relative risks, based on a negative heterogeneity test, and this may be inappropriate as each of the included studies have varied populations, subject selection criteria and treatment protocols. A lack of statistical heterogeneity may have been demonstrated by the authors, however, significant clinical heterogeneity left room for doubt in the conclusions of this meta-analysis.

The publication attracted several letters to the publishing journal (JAMA) criticizing methodological and other aspects of the study. Levien et al. mentioned that a study of 336 patients conducted in South Africa examining Hemopure was not included in the metanalysis [7]. These authors also emphasized that the use of HBOCs should be tailored to specific populations of patients where banked blood is not an option, for example Jehovah's witnesses and patients with severe autoimmune hemolytic anemia. Further rebukes included the correction of inaccurate or missing trial data [8, 9], explanations for delay in publicising negative trial findings [10], and arguments for careful continuation of clinical trials investigating HBOCs [11–13].

Despite these and other objections, the study conclusion was considered by the FDA in a scheduled conference held soon after the online publication and a decision was made by the regulatory authority to apply "careful weighing of potential risks and benefits" prior to allowing any further HBOC clinical trials to proceed. Subsequently, the FDA placed a hold on any further clinical trial involving an HBOC.

Another confounding issue implied in the Natanson article is that the majority (up to 75%) of data for Hemopure in particular, was not published and was instead retrieved from minutes of an FDA hearing [5]. There may be valid concern that positive publication bias based on commercial interests, and FDA delays, may play a significant role in the public data available for analysis of the utility and safety of HBOCs. Citing public interest concerns about testing HBOCs on civilian trauma patients, a public advocacy group called "Public Citizen" sued the FDA in order to open to the public a meeting in which a discussion about blood substitutes, in particular Hemopure, was scheduled to take place [14]. In later interviews, Natanson also claimed that the manufacturers were hesitant to release data, and only the threat of legal action would make them do so.

The publication of the Natanson meta-analysis resonated through the academic world when Biopure, the manufacturer of Hemopure, sued Dr Charles Natanson for trade libel, defamation and intentional interference in 2008 [15]. Further intensifying debate was the fact that Dr Natanson failed to disclose a key conflict of interest [16]. The suit was eventually withdrawn, and Biopure was acquired by HBO2 Therapeutics.

The study of HBOCs is complex. Factors such as the molecular characteristics of the compound and individual patient factors need to be accounted for. Just as synthetic colloids are recognized as separate compounds and assessed independently, so should HBOCs. It is clear that any study examining the "safety" issues of HBOCs should be sufficiently well designed and powered, with meticulous attention paid to patient characteristics, compound pharmacology and ethical principles.

Safety Aspects of Specific HBOCs

HemAssist

In the 1980s the Letterman Army Institute of Research (LAIR) coupled with the Baxter company created the Diaspirin cross-linked human hemoglobin named HemAssist. It is a stroma-free human hemoglobin intramolecularly cross-linked between the α-chains of the tetrameric molecule. The LAIR sponsored studies found discouraging results reporting vasoconstriction related adverse events, and after

some disagreements, severed ties with Baxter. Baxter pursued investigation of the product in Phase I, II and eventually Phase III clinical trials. They reported a significant increase in venous red blood cell velocity, a constriction of arterioles for a period of 2 minutes, a significant change in arteriolar vasomotion frequency and amplitude at different sites of the arteriolar tree and absence of microvascular disturbances. In addition, Baxter found a relevant elevation of the mean arterial pressure after HemAssist infusion accompanied by a reflex bradycardia [17]. Six clinical trials that evaluated the HemAssist reported contradictory results in terms of vasoconstriction, stroke and survival [18–23]. In 1997 Baxter launched its own clinical study on patients in transported from the scene in an ambulance or on arrival to the emergency department [22]. The data safety monitoring committee advised that HemAssist was found to be significantly less effective than the standard of care, and the study was terminated. The data showed that 46% of patients who received HemAssist died versus 17% of patients receiving 0.9% saline. Finally, another European study in patients with severe hemorrhagic shock evaluating the usefulness of HemAssist in preventing multiorgan failure (MOF) from tissue hypoxia was also unsuccessful in showing any benefit [23]. The study was ceased before completion together with the manufacture of the product.

Optro

In 1992, Somatogen reported the production of Optro(rHb1.1), a recombinant human hemoglobin for clinical use [24]. It is a genetically engineered protein produced by *E. coli* consisting of a tetramer of two alpha-globin peptides, with a longer half-life (therefore reducing kidney injury) and reduced oxygen affinity than other earlier cell-free hemoglobins. The company entered a partnership with the pharmaceutical company Eli-Lilly. Initial small clinical studies showed relative safety with self-limiting hypertension, pyrogenicity, mildly elevated bilirubin, amylase and liver enzymes being the main adverse effects [25]. Renal function was not affected. Hypertension was quite noteworthy, for example in the most severe case, mean arterial pressure rose to 144 mmHg. Subsequent Phase II clinical trials demonstrated similar side effects. Eli-Lilly ended the partnership, and further clinical trials were halted [26].

Hemolink

Hemolink is a O-raffinose cross-linked and polymerised human hemoglobin developed by Hemosol Biopharma (Mississauga, Canada). It was developed as a potential blood replacement during cardiopulmonary bypass and other settings, during which the patient's own blood could be saved for later re-transfusion. Hill et al., conducted a phase II clinical trial including 60 patients undergoing CABG [27]. Patients were randomized to receive either hemoglobin raffimer or hetastarch (6%) in 0.9% sodium chloride. Patients receiving Hb raffimer had more hypertension, jaundice and elevation of AST and lipase. Serious adverse events were equal in each group. There was a reduction in the volume of RBC transfusion in the Hb raffimer group. Cheng et al. demonstrated a similar outcome in a phase II dose-response study also examining a group of patients undergoing CABG [28]. In a phase III trial, 299 CABG patients were enrolled and although the Hemolink group had fewer blood transfusions, there was a 10% greater incidence of adverse events compared with controls [29]. These adverse events included hypertension, non-significant increase in myocardial infarcts, jaundice, elevated liver and pancreatic enzymes and abnormal urine colour. Despite these initial promising results, further trials were halted in part related to an increased rate of cardiac events in patients receiving Hemolink.

Polyheme

Polyheme is a human hemoglobin was developed by Northfield Laboratories. It was derived from human blood cells and presented in an electrolyte solution. The first study evaluating the effects of Polyheme when compared to allogenic blood transfusion in trauma patients demonstrated that Polyheme maintained hemoglobin concentration and reduced the need for allogenic blood by 3.5 units [30]. Another study, comparing Polyheme to a historical control group was deemed unsuitable by the Food and Drug Administration (FDA) [31]. The FDA was reluctant to accept a historical control data group for a Phase III clinical trial study. A further phase III trial was announced in December 2006 and by May 2007, 586 patients with traumatic hemorrhagic shock were included in the study and were treated with either Polyheme (279 patients) or hospital standard of care (307 patients) [32, 33]. A 30-day mortality follow-up found 88.9% survival in the Polyheme group versus 90.9% in the control group. With respect to adverse effects, 93% in the Polyheme group versus 88% in the control group experienced at least one of anemia, fever or electrolyte imbalance. In addition, there were several issues with the final IRB approval for the study. The absence of a requirement in the trial to administer allogenic blood to victims upon arrival was considered unethical by the reviewing committee. In May 2009, the FDA refused to approve Polyheme and in June of that year the Northfield Laboratories company filed for bankruptcy protection.

MP40x-Hemospan ("3rd Generation")

Hemospan, later called MP40x, was developed by Sangart as an "oxygen therapeutic" rather than a complete blood substitute. It was a maleimide conjugated human hemoglobin, with a larger size and increased oxygen affinity. These properties promised to allow increased intravascular retention time and normal intravascular tone resulting in increased oxygen delivery to at-risk tissues [34]. This compound was mainly investigated in the context of elective orthopedic surgery. Initial studies were promising; Olofsson et al. conducted a study of 90 patients undergoing elective hip surgery, and although there was a mild elevation in liver enzymes and lipase, there were no serious adverse events in the MP40x group [35]. A further dose response study in a similar population group showed mild to moderate adverse events such as nausea, methemoglobinemia and elevation of liver enzymes [36]. There were two patients who had a transient elevation of troponin, but no ECG changes and were asymptomatic. Only one patient was reported to have elevated blood pressure after administration. Olofsson et al. then conducted a multicenter Phase III randomized controlled study including 367 patients undergoing elective primary hip arthroplasty. Patients were randomized to receive either hydroxyethyl starch or MP40x. The study found that although MP40x reduced the incidence of hypotension, there were more adverse events such as hypertension, elevation of liver enzymes, elevation of lipase and troponin. There were no serious adverse events noted, but the authors concluded that the adverse event profile did not warrant the use of MP40x in the population group studied [4]. Sangart subsequently terminated production of the product.

Hemopure (Biopure, OPK Pharma, HBO2 Therapeutics)

Hemopure (hemoglobin glutamer 250, HBOC-201) is a highly purified glutaraldehyde-polymerized bovine hemoglobin. The initial inception product, hemoglobin glutamer 301/200 (HBOC-200) received veterinary approval in the US (1997) and Europe (1998). HBOC-201, the subsequently developed human product, only received approval for human use in acute surgical hemorrhage in South Africa in 2001 and for acute, all-cause anemia in Russia in 2010. HBOC-201 is also available in the United States under the FDA "Expanded Access" category.

Therefore, despite the controversy surrounding the Natanson meta-analysis publication and its chilling effect on all HBOC development, Hemopure remains the most widely studied HBOC. As a result, there are several case series, observational studies and randomized controlled trials demonstrating safety and efficacy of the compound in certain clinical situations. HBOC-201 stability ranges between 18 months to 3 years, depending on the preservation temperature and cross-matching is not required. In addition, it is sterile and ready for immediate use. The most common adverse effects include hypertension, methemoglobinemia, elevated liver enzymes and bilirubin, volume overload, headaches, abdominal cramps, achalasia and headache [11, 37–47]. These adverse effects were labelled as transient or easily treatable in most cases.

The largest randomized controlled HBOC trial was published in 2008. In the "HEM-0115" trial, Jahr et al. randomized 693 patients undergoing elective orthopedic surgery into two groups, those receiving Hemopure and those receiving packed red cells after certain clinical transfusion requirement criteria had been met [48]. Patients were followed for 6 weeks postoperatively. The HBOC group had 977 more adverse events. However, many of these adverse events were ascribed by the authors to be known physiological effects of HBOC-201. Adverse events noted were skin discoloration, gastrointestinal side effects, transient mild hypertension and transiently raised liver enzyme and lipase levels. The HBOC-201 group had 35 more severe adverse events than the PRBC group and cardiac related events and strokes were higher in the HBOC-201 group. Further analysis within the paper proposed three risk factors resulting in greater serious adverse events in the study group: (1). Patient age, (2). Volume overload, and (3). Undertreatment. Patients over 80 years old constituted a large proportion of patients who developed cardiac events. Mortality was not different between the study arms.

The findings of this randomized control trial and other unpublished HBOC-201 data was pooled in the 2008 FDA analysis, and the higher cumulative risk of cardiac and cerebrovascular events lead to the suspension of further study approval.

Van Hemelrijck et al. reported in 2014 previously unpublished data from a randomized controlled trial conducted in 1998–1999. The authors describe a trial of HBOC-201 use versus packed red blood cell transfusion in a non-cardiac surgery population. Adverse events such as hypertension, dysphagia, nausea, abdominal pain and skin discolouration were more common in the HBOC-201 arm. However, the risk of serious adverse events, in particular cardiac events and mortality, were not significantly different between the two groups [49].

Most recently, in 2019 Mackenzie et al., published a review combining data of 1701 clinical uses of HBOC-201, incorporating data from case series and the randomized control trials mentioned above [50]. Common adverse events identified were transiently increased blood pressure, jaundice, methemoglobinaemia, increased liver and pancreatic enzymes, decreased pulse oximetry measurements and oli-

guria. All events were considered as non-serious and transient. There was no cardiotoxicity noted.

Finally, data from clinical use in South Africa have yielded "consensus usage guidelines" by a group of clinical experts using Hemopure in practice [51]. The publication includes guidance about indications, contra-indications, dosage and administration, monitoring and treatment of side-effects. Of note is that the authors precaution against the use of Hemopure in patients over the age of 80, those with cardiac disease and those with volume overload. These were the risk factors identified for serious adverse events in the Jahr 2008 randomized controlled trial.

A review by Jahr et al. [52] reported six studies comparing the cardiac outcomes of HBOC-201 versus colloids and albumin in animal models. HBOC-201 was reported to decrease the incidence of myocardial necrosis after myocardial infarction by 50%. In addition, there was an increased capacity to restore the systemic pressure and oxygen delivery, no decrease in issue oxygenation and no alteration in cardiac performance. HBOC-201 had no effect on myocardial shortening, myocardial stroke work, and post-occlusion reactive hyperemia. One of these studies [53], reported an increase in brain and kidney tissue oxygenation, no change in methemoglobin levels, and no change in cardiac hemodynamics compared to human serum albumin. Another animal study [54] highlighted the difference in outcomes in HBOC-201 and a control group using a dog model. Platelet count and hematocrit level were higher in the HBOC-201 group at 1 hour after the infusion. In addition, there was a significant reduction of myocardial enzyme levels and a 56% decreased infarct size in the HBOC-201 group. A similar trend in outcomes was also reported by another group [55] demonstrating a beneficial preventive effect of HBOC-201 in ischemia induced decrease in mean aortic blood pressure, stroke volume, cardiac output, left ventricular systolic pressure, and alterations in regional wall motion abnormalities. When comparing outcomes between blood and HBOCs Ortiz et al. [56], in another animal model, found HBOC-201 tends to release oxygen at a higher pO_2 level compared to blood. With respect to the mean arterial pressure, there were no differences in between the two groups. Blood produced a significant rise in pCO_2, while HBOCs had a higher oxygen extraction ratio and greater restoration of arterial diameter. Similar outcomes were also reported by Mongan et al. [57] who studied HBOC-201 versus human serum albumin in saline. The HBOC-201 group experienced a significantly higher mean arterial pressure, higher systemic and pulmonary vascular resistance, lower venous pO_2, but no difference in organ perfusion and tissue vascular resistance. Finally, these animal studies have been supported by clinical data. A study examining the safety of HBOC-201 in the setting of elective percutaneous coronary intervention (for patients with stable angina and non-ST-segment elevation myocardial infarction) randomized patients into 3 groups; 230 mL HBOC-201, 115 mL HBOC-201, and the control arm received 230 mL synthetic colloid (Voluven-Fresenius) [58]. Hypertension was seen in both HBOC-201 groups. However, there was determined to be no vasoconstrictive effect of HBOC-201 on coronary vasculature and no increase in left ventricular stroke work.

Cognizant of the existing and emerging data on HBOC efficacy and safety in certain clinical contexts, the FDA in conjunction with the NIH and US military, agreed in a 2017 meeting that further research and development into HBOC development should be considered [59].

A collaborative effort between the US Department of Defense and Stellenbosch University in South Africa is underway to evaluate the use of Hemopure and freeze-dried plasma in the pre-hospital civilian trauma setting in South Africa [60]. The study is being conducted across 21 hospitals and 27 ambulance bases; and hopes to recruit 1400 patients over 3 years. Since the compound is approved in South Africa, there is little difficulty in designing an ethical trial based on good clinical practice principles. Pre-hospital trials in jurisdictions such as the US face the barrier of ethical uncertainty in the setting of administering a non-approved drug to a patient who is unable to consent.

Sanguinate (Prolong Pharmaceuticals)

Sanguinate is a recently developed pegylated bovine derived carboxyhemoglobin molecule produced by Prolong Pharmaceuticals. It is engineered to release carbon monoxide(CO), as well as carry oxygen [61]. This unique characteristic ameliorates the NO scavenging property of hemoglobin-based oxygen carriers by the release of CO. CO release causes vasodilatation and has been demonstrated to have anti-inflammatory effects [62]. An initial phase I clinical study with varying dosage groups demonstrated a favorable safety profile, with no serious adverse events noted in health volunteers [63]. However, in addition to non-specific adverse events such as lethargy and dizziness, mild transient hypertension was noted. The hypertension resolved without treatment within 72 hours. A reduction in haptoglobin in all groups was seen, and this reflects the normal homeostatic mechanism of hemoglobin removal. Due to the vasodilatory properties of CO, the target population for therapeutic use of Sanguinate has been patients with sickle cell disease (SCD) experiencing a sickle crisis, which is characterized by severe localized vasoconstriction and vaso-occlusion. Misra et al. conducted a phase Ib clinical trial in SCD patients not currently in crisis [64]. In the group of patients receiving Sanguinate, transient hypertension was noted. The most common adverse event was arthralgia and muscle pain, reported in 75% of patients who received Sanguinate. Of

most concern was a rise in serum troponin I levels, which was noted in 25% of patients receiving Sanguinate. These patients were asymptomatic and the enzyme rise was transient. Adverse events were more common in the higher dosage group. The authors concluded that the compound was safe to use in stable SCD patients. Sanguinate is still undergoing several clinical trials, and has not been licensed for clinical use, but it has been made available via the FDA "expanded access" category, and there are published case studies supporting its use and safety profile. Jehovah's witness patients have used Sanguinate as an alternative to blood transfusion. Examples of clinical situations where Sanguinate has been used include cases of surgical hemorrhage, severe sepsis, thrombotic cytopenic purpura and postpartum hemorrhage [65–69]. As described previously, the most common side effects include transient hypertension, musculoskeletal pain and troponin rise.

Adverse Events

Although free hemoglobin oxygen carriers were judged to have class-based effects in the Natanson meta-analysis and subsequent FDA ruling, it has become clear that although similar adverse events are seen with each generation and variation of HBOC, the extent of these various adverse events differ significantly between compounds. Common to most HBOCs are possible adverse events such as hypertension, achalasia, nausea, abdominal pain, skin discolouration, elevated liver enzymes, elevated pancreatic enzymes and volume overload. More rare, serious adverse events such as myocardial infarction, cardiac failure, arrhythmias and cerebrovascular accidents have been described [70, 71], but they tend to be patient and product specific. Blood oxygen desaturation has also been noted, but this is usually as a result of concomitant cardiac failure, or inaccurate oximeter readings secondary to the oxygen binding characteristics of HBOCs. See Table 13.1 for a summary of adverse effects and possible therapies.

These effects are likely mediated by HBOC associated nitric oxide scavenging, hyperoxygenation and heme breakdown related toxicity. Reduced available nitric oxide and hyperoxygenation mediated disruption of autoregulation can result in vasoconstriction leading to a rise in blood pressure, and reduced organ blood flow. A reduction in cardiac output can be seen with increasing systemic vascular resistance and blood pressure. A concomitant reduction in oxygen delivery is possible. This explains the common blood pressure elevating response seen with the infusion of these agents. Hypertension is rarely severe and has been treated with calcium channel blockers or beta blockers.

Table 13.1 Potential adverse effects of HBOCs and their Management

Adverse effect	Mechanism	Management
Hypertension (systemic and Pulmonary)	Nitric oxide scavenging, loss of vascular autoregulation [3, 70]	Antihypertensives – CCBs, beta blockers, nitrates
Methaemoglobinemia	NADPH reductase levels overwhelmed and Met Hb increases [43, 70]	Administration of ascorbic acid or methylene blue if more than 15%
Achalasia	Impaired relaxation of lower oesophageal sphincter. SMC contraction due to NO scavenging [41]	Anticholinergic administration [51]
Fluid overload	Iso-oncotic expander [43, 48]	Stop infusion, diuresis, vasodilator
Liver enzyme increase	Possible colorimetric interference [43, 74]	Observe and repeat test
Decreased cardiac output	Volume overload and vasoconstriction [43, 71]	Stop infusion, diuresis, vasodilator, inotrope
Decrease in pulse oximetry (5–10%)	Shift of HB-O2 curve [51]	Arterial blood gas to confirm. Usually transient
Skin/urine darkening	Breakdown of free hemoglobin	Observe and treat symptomatically with antihistamine

However, although it may be possible that reduced oxygen delivery may be related to serious adverse events such as myocardial infarction and strokes, there is data to dispute this supposition [72], suggesting that a more complex interplay occurs between the HBOC and already pre-existing dysfunctional endothelium. Hare et al. [73] reported a significant beneficial increase in regional cortical cerebral blood flow and no changes in the caudate tissue oxygen tension after pentastarch administration to animal models.

Nitric oxide consumption is responsible for other smooth muscle contraction, particularly in the gastrointestinal tract resulting in achalasia, nausea and abdominal pain. There is no literature suggesting that this could progress to ischemia or perforation.

Breakdown of free hemoglobin may lead to an increased porphyrin load, bilirubin and cutaneous icterus. In addition, hemoglobin outside of a cellular membrane is more prone to oxidation and conversion to methemoglobin due to consumption of existing NADPH reductase. Methemoglobinemia has possible cardiac, metabolic and neurological effects, particularly if levels are greater than 50%. Although methemoglobinemia occurs after infusion of all free hemoglobin blood substitutes, the level rarely exceeds 15–20%, and is easily treatable. Ascorbic acid has been used as therapy to

avert the effects of free hemoglobin oxidation successfully [40]. In severe cases of methemoglobinemia, methylene blue infusion can be used.

Volume overload can increase cardiac strain and therefore the risk of myocardial ischemia and arrhythmias. Since HBOCs are considered to be hyperoncotic, slow infusion is recommended, and the presence of pre-existing fluid overload represents a contraindication to their use. Unlike previous preparations of HBOCs, second and third generations do not have an apparent effect on kidney function, with most studies demonstrating no obvious renal toxicity.

Finally, liver and pancreatic enzyme levels are often transiently raised, without other features of hepatitis or pancreatitis. This effect is not well elucidated, and it may be as a result of laboratory instrument error proportional to the amount of free hemoglobin present in the plasma [74]. Regardless, there has been no report of persisting pancreatic or liver dysfunction in the literature.

Conclusion

There is an ever-increasing need for donated blood in prehospital and hospitalized patients with acute anemia. Banked blood supplies fluctuate unpredictably and are often completely unavailable in certain remote settings such as the battlefield, or even in most out-of-hospital civilian trauma environments. There are also situations in which allogeneic blood is contraindicated, for example severe autoimmune hemolytic anemia, or in religious circumstances such as Jehovah's witnesses. Hemoglobin-based oxygen carriers are promising potential alternatives to allogeneic blood transfusion, which can lead to restoration of blood volume, organ perfusion and oxygen delivery. Unfortunately, since the late 1990s, when HBOCs were first clinically apparent in the literature, these compounds have been fraught with setbacks. Initial stroma free hemoglobins had adverse renal effects and caused significant hypertension. Subsequent generations of polymerized hemoglobins are larger molecules, which have longer half lives and less renal complications. The rise in blood pressure, which is secondary to nitric oxide consumption and vasculo-endothelial dysregulation, although easy enough to manage, may be an issue. In addition, the hyperoncotic nature of the drug can result in volume expansion and overload in susceptible patients. Hypertension and hypervolemia in at risk individuals can lead to cardiac events, such as arrhythmias, myocardial infarction, cardiac failure and neurologic events. Other possible adverse events such as hepatic and pancreatic enzyme elevation, skin and urine discolouration, and methemoglobinemia are transient, and seem to have no long-term effect. Although these minor adverse effects are relatively manageable and non-significant in patients without risk factors for cardiac disease, regulatory authorities have been reluctant to approve investigation of the products in randomized controlled trials. Blood transfusion has risks, but these are relatively rare and well accepted. It is therefore now more difficult to design an ethically acceptable study randomizing surgical or trauma patients to either receive an HBOC or blood, when the gold standard (donated blood) is so available and apparently carries a lower risk compared with the test drug. Although areas of need, such as the battlefield, may be more ethically acceptable in which to conduct such a study, the risk of imprecise or incomplete data collection is high. Another option is studying HBOCs in populations that can only use HBOCs, such as severe autoimmune hemolytics or Jehovah's witnesses, but this would not yield a large enough sample size to be statistically useful.

Unfortunately, regulatory authorities have historically grouped together the entire class of HBOCs, disregarding the varying spectrum of effects and adverse events across compound configurations and generations. This resulted in a hiatus of clinical investigation into HBOCs particularly from 2008 to 2015. However, HBOC-201 (Hemopure) is being used in South Africa and Russia where it is licensed for clinical use, as well as in the United States under the FDA "compassionate use" or "expanded access" category. In addition, Sanguinate is also a promising molecule, with a favorable safety profile, which is currently undergoing early clinical investigation. Many case reports and series have been published, demonstrating safety and clinical efficacy of these drugs.

Accumulation of these reports, as well as further Phase I trials demonstrating pharmacodynamic mechanisms, have opened the door again for further research into HBOCs.

References

1. Points to consider in the safety evaluation of hemoglobin-based oxygen carriers. Center for Biologics Evaluation and Research. Transfusion. 1991;31(4):369–71.
2. Mackenzie CF. Haemoglobin-based oxygen carriers: is the benefit worth the risk? Br J Hosp Med. 2009;70(1):26–30.
3. Taverne YJ, de Wijs-Meijler D, Te Lintel HM, Moon-Massat PF, Dube GP, Duncker DJ, et al. Normalization of hemoglobin-based oxygen carrier-201 induced vasoconstriction: targeting nitric oxide and endothelin. J Appl Physiol. 1985;122(5):1227–37.
4. Olofsson CI, Górecki AZ, Dirksen R, Kofranek I, Majewski JA, Mazurkiewicz T, et al. Evaluation of MP4OX for prevention of perioperative hypotension in patients undergoing primary hip arthroplasty with spinal anesthesia: a randomized, double-blind, multicenter study. Anesthesiology. 2011;114(5):1048–63.
5. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA. 2008;299(19):2304–12.
6. Perel P, Roberts I, Ker K. Colloids versus crystalloids for fluid resuscitation in critically ill patients. Cochrane Database Syst Rev. 2013;(2):Cd000567.
7. Levien LJ. Bovine hemoglobin (hemopure): clinical use in South Africa. Vox Sang. 2006;91:22.

8. Keipert PE, Olofsson C, Winslow RM. Hemoglobin-based blood substitutes and risk of myocardial infarction and death. JAMA. 2008;300(11):1295–9.
9. Shander A, Javidroozi M, Thompson G. Hemoglobin-based blood substitutes and risk of myocardial infarction and death. JAMA. 2008;300(11):1295–9.
10. Lewis RJ, Fost N. Hemoglobin-based blood substitutes and risk of myocardial infarction and death. JAMA. 2008;300(11):1295–9.
11. Donahue LL, Shapira I, Shander A, Kolitz J, Allen S, Greenburg G. Management of acute anemia in a Jehovah's witness patient with acute lymphoblastic leukemia with polymerized bovine hemoglobin-based oxygen carrier: a case report and review of literature. Transfusion. 2010;50(7):1561–7.
12. Sauaia A, Moore EE, Banerjee A. Hemoglobin-based blood substitutes and risk of myocardial infarction and death. JAMA. 2008;300(11):1295–9.
13. Sarani B, Gracias V. Hemoglobin-based blood substitutes and risk of myocardial infarction and death. JAMA. 2008;300(11):1295–9.
14. Citizen P. Wolfe v. FDA. 2006. Available from: https://www.citizen.org/litigation/sidney-wolfe-v-fda/.
15. Ledford H. Company sues researcher over unfavourable review Nature. 2008. Available from: https://www.nature.com/news/2008/081111/full/news.2008.1219.html.
16. Natanson C. Incomplete financial disclosure in a study of cell-free hemoglobin-based blood substitutes and risks of myocardial infarction and death. JAMA. 2008;300(11):1300.
17. Nolte D, Steinhauser P, Pickelmann S, Berger S, Härtl R, Messmer K. Effects of diaspirin-cross-linked hemoglobin (DCLHb) on local tissue oxygen tension in striated skin muscle: an efficacy study in the hamster. J Lab Clin Med. 1997;130(3):328–38.
18. Lamy ML, Daily EK, Brichant JF, Larbuisson RP, Demeyere RH, Vandermeersch EA, et al. Randomized trial of diaspirin cross-linked hemoglobin solution as an alternative to blood transfusion after cardiac surgery. The DCLHb cardiac surgery trial collaborative group. Anesthesiology. 2000;92(3):646–56.
19. Saxena R, Wijnhoud AD, Carton H, Hacke W, Kaste M, Przybelski RJ, et al. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke. Stroke. 1999;30(5):993–6.
20. Schubert A, Przybelski RJ, Eidt JF, Lasky LC, Marks KE, Karafa M, et al. Diaspirin-crosslinked hemoglobin reduces blood transfusion in noncardiac surgery: a multicenter, randomized, controlled, double-blinded trial. Anesth Anal. 2003;97(2):323–32.
21. Sloan EP, Koenigsberg M, Gens D, Cipolle M, Runge J, Mallory MN, Rodman G Jr., et al. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial. (0098–7484 (Print)).
22. Corporation BH. Baxter Ends U.S. Trauma Study of HemAssist(TM) (DCLHb) 1998 [cited 2021 22 March]. Available from: http://web.archive.org/web/19981205122026/dclhb.er.uic.edu/press_release.htm.
23. Kerner T, Ahlers O, Veit S, Riou B, Saunders M, Pison U, et al. DCL-Hb for trauma patients with severe hemorrhagic shock: the European "on-scene" multicenter study. Intensive Care Med. 2003;29(3):378–85.
24. Looker D, Abbott-Brown D, Cozart P, Durfee S, Hoffman S, Mathews AJ, et al. A human recombinant haemoglobin designed for use as a blood substitute. Nature. 1992;356(6366):258–60.
25. Hayes JK, Stanley TH, Lind GH, East K, Smith B, Kessler K. A double-blind study to evaluate the safety of recombinant human hemoglobin in surgical patients during general anesthesia. J Cardiothorac Vasc Anesth. 2001;15(5):593–602.
26. Brower V. Blood products have future despite recent setbacks. Nat Biotechnol. 1997;15(6):507.
27. Hill SE, Gottschalk LI, Grichnik K. Safety and preliminary efficacy of hemoglobin raffimer for patients undergoing coronary artery bypass surgery. J Cardiothorac Vasc Anesth. 2002;16(6):695–702.
28. Cheng DC, Mazer CD, Martineau R, Ralph-Edwards A, Karski J, Robblee J, et al. A phase II dose-response study of hemoglobin raffimer (Hemolink) in elective coronary artery bypass surgery. J Thorac Cardiovasc Surg. 2004;127(1):79–86.
29. Greenburg AG, Kim HW. Use of an oxygen therapeutic as an adjunct to intraoperative autologous donation to reduce transfusion requirements in patients undergoing coronary artery bypass graft surgery. J Am Coll Surg. 2004;198(3):373–83; discussion 84–5.
30. Gould SA, Moore EE, Hoyt DB, Burch JM, Haenel JB, Garcia J, DeWoskin R, et al. The first randomized trial of human polymerized hemoglobin as a blood substitute in acute trauma and emergent surgery. (1072–7515 (Print)).
31. Gould SA, Moore EE, Hoyt DB, Ness PM, Norris EJ, Carson JL, et al. The life-sustaining capacity of human polymerized hemoglobin when red cells might be unavailable. J Am Coll Surg. 2002;195(4):445–52; discussion 52–5.
32. States U, Commission SAE. For annual and transition reports pursuant to section 13 OR 15(d) of the Securities Exchange Act of 1934: Commission file number 0–24050 Northfield Laboratories Inc. 2007 [cited 2021 22 March]. Available from: https://sec.report/Document/0000950137-07-012373/.
33. Release. NLP. Northfield Laboratories reports results of pivotal Phase III trauma study 2007 [cited 2021 22 March]. Available from: https://www.marketscreener.com/quote/stock/NORTHFIELD-LABORATORIES-10188/news/Northfield-Labs-nbsp-Northfield-Laboratories-Reports-Results-of-Pivotal-Phase-III-Trauma-Study-367744/.
34. Vandegriff KD, Winslow RM. Hemospan: design principles for a new class of oxygen therapeutic. Artif Organs. 2009;33(2):133–8.
35. Olofsson C, Ahl T, Johansson T, Larsson S, Nellgård P, Ponzer S, et al. A multicenter clinical study of the safety and activity of maleimide-polyethylene glycol-modified hemoglobin (Hemospan) in patients undergoing major orthopedic surgery. Anesthesiology. 2006;105(6):1153–63.
36. Olofsson C, Nygårds EB, Ponzer S, Fagrell B, Przybelski R, Keipert PE, et al. A randomized, single-blind, increasing dose safety trial of an oxygen-carrying plasma expander (Hemospan) administered to orthopaedic surgery patients with spinal anaesthesia. Transfus Med. 2008;18(1):28–39.
37. Stefan DC, Uys R, Wessels G. Hemopure transfusion in a child with severe anemia. Pediatr Hematol Oncol. 2007;24(4):269–73.
38. Pachinburavan M, Marik PE. Bovine blood and neuromuscular paralysis as a bridge to recovery in a patent with severe autoimmune hemolytic anemia. CTS Clin Transl Sci. 2008;1(2):172–3.
39. Marinaro J, Smith J, Tawil I, Billstrand M, Crookston KP. HBOC-201 use in traumatic brain injury: case report and review of literature. Transfusion. 2009;49(10):2054–9.
40. Fitzgerald MC, Chan JY, Ross AW, Liew SM, Butt WW, Baguley D, et al. A synthetic haemoglobin-based oxygen carrier and the reversal of cardiac hypoxia secondary to severe anaemia following trauma. Med J Aust. 2011;194(9):471–3.
41. Epperla N, Strouse C, VanSandt AM, Foy P. Difficult to swallow: warm autoimmune hemolytic anemia in a Jehovah's witness treated with hemoglobin concentrate complicated by achalasia. Transfusion. 2016;56(7):1801–6.
42. Posluszny JA, Napolitano LM. Hemoglobin-based oxygen carrier for traumatic hemorrhagic shock treatment in a Jehovah's witness. Arch Trauma Res. 2016;5(2):3.
43. Gomez MF, Aljure O, Ciancio G, Lynn M. Hemoglobin-based oxygen carrier rescues double-transplant patient from life-threatening anemia. Am J Transplant. 2017;17(7):1941–4.
44. Davis JM, El-Haj N, Shah NN, Schwartz G, Block M, Wall J, et al. Use of the blood substitute HBOC-201 in critically ill patients during sickle crisis: a three-case series. Transfusion. 2018;58(1):132–7.

45. Unnikrishnan A, Pelletier JPR, Bari S, Zumberg M, Shahmohamadi A, Spiess BD, et al. Anti-N and anti-do(a) immunoglobulin G alloantibody-mediated delayed hemolytic transfusion reaction with hyperhemolysis in sickle cell disease treated with eculizumab and HBOC-201: case report and review of the literature. Transfusion. 2019;59(6):1907–10.
46. Rubinstein MM, Goss C, Avecilla ST, Dub GP, Riely GJ, Mones JV. Management of thymoma-associated pure red cell aplasia: a novel use of blood substitute HBOC-201 in a Jehovah's witness. Clin Case Rep. 2020;8(2):289–92.
47. Zumberg M, Gorlin J, Griffiths EA, Schwartz G, Fletcher BS, Walsh K, et al. A case study of 10 patients administered HBOC-201 in high doses over a prolonged period: outcomes during severe anemia when transfusion is not an option. Transfusion. 2020;60(5):932–9.
48. Jahr JS, Mackenzie C, Pearce LB, Pitman A, Greenburg AG. HBOC-201 as an alternative to blood transfusion: efficacy and safety evaluation in a multicenter phase III trial in elective orthopedic surgery. J Trauma. 2008;64(6):1484–97.
49. Van Hemelrijck J, Levien LJ, Veeckman L, Pitman A, Zafirelis Z, Standl T. A safety and efficacy evaluation of hemoglobin-based oxygen carrier HBOC-201 in a randomized, multicenter red blood cell controlled trial in noncardiac surgery patients. Anesth Analg. 2014;119(4):766–76.
50. Mackenzie CF, Dube GP, Pitman A, Zafirelis M. Users guide to pitfalls and lessons learned about HBOC-201 during clinical trials, expanded access, and clinical use in 1,701 patients. Shock. 2019;52:92–9.
51. Mer M, Hodgson E, Wallis L, Jacobson B, Levien L, Snyman J, et al. Hemoglobin glutamer-250 (bovine) in South Africa: consensus usage guidelines from clinician experts who have treated patients. Transfusion. 2016;56(10):2631–6.
52. Jahr JS, Tseng K, Brown AP, Dube' G. Hemoglobin-glutamer 250 (bovine) [HBOC-201, Hemopure®] Clinical use in South Africa and worsened or improved cardiac outcomes-a comprehensive review of risk/benefit in peer-reviewed, indexed studies in humans and animal models. (Chapter for Nanobiotherapeutic based Blood Substitutes, Edited by Chang TMS, Bulow L, Jahr J, Sakai H, Yang C). 2021;207–47
53. Muir WW, Ilangovan G, Zweier JL, Moon-Massat PF, Rentko VT. Vital organ tissue oxygenation after serial normovolemic exchange transfusion with HBOC-201 in anesthetized swine. Shock. 2011;35(6):597–603.
54. Caswell JE, Strange MB, Rimmer DM 3rd, Gibson MF, Cole P, Lefer DJ. A novel hemoglobin-based blood substitute protects against myocardial reperfusion injury. Am J Physiol Heart Circ Physiol. 2005;288(4):H1796–801.
55. Te Lintel HM, Dubé GP, Regar E, de Boer M, Vranckx P, van der Giessen WJ, et al. Preoxygenated hemoglobin-based oxygen carrier HBOC-201 annihilates myocardial ischemia during brief coronary artery occlusion in pigs. Am J Physiol Heart Circ Physiol. 2010;298(3):H1103–13.
56. Ortiz D, Barros M, Yan S, Cabrales P. Resuscitation from hemorrhagic shock using polymerized hemoglobin compared to blood. Am J Emerg Med. 2014;32(3):248–55.
57. Mongan PD, Moon-Massat PF, Rentko V, Mihok S, Dragovich A, Sharma P. Regional blood flow after serial normovolemic exchange transfusion with HBOC-201 (Hemopure) in anesthetized swine. J Trauma. 2009;67(1):51–60.
58. Serruys PW, Vranckx P, Slagboom T, Regar E, Meliga E, de Winter RJ, et al. Haemodynamic effects, safety, and tolerability of haemoglobin-based oxygen carrier-201 in patients undergoing PCI for CAD. EuroIntervention. 2008;3(5):600–9.
59. Khalili R. Future blood: tools and technology for the battlefield and beyond. 2017. Available from: https://www.army.mil/article/182662/future_blood_tools_and_technology_for_the_battlefield_and_beyond.
60. Stellenbosch Uo. SU awarded contract to direct large clinical study in trauma resuscitation. 2018. Available from: https://www.sun.ac.za/english/Lists/news/DispForm.aspx?ID=6060.
61. Abuchowski A. PEGylated bovine carboxyhemoglobin (SANGUINATE™): results of clinical safety testing and use in patients. Adv Exp Med Biol. 2016;876:461–7.
62. Abuchowski A. SANGUINATE (PEGylated carboxyhemoglobin bovine): mechanism of action and clinical update. Artif Organs. 2017;41(4):346–50.
63. Misra H, Lickliter J, Kazo F, Abuchowski A. PEGylated carboxyhemoglobin bovine (SANGUINATE): results of a phase I clinical trial. Artif Organs. 2014;38(8):702–7.
64. Misra H, Bainbridge J, Berryman J, Abuchowski A, Galvez KM, Uribe LF, et al. A phase Ib open label, randomized, safety study of SANGUINATE™ in patients with sickle cell anemia. Rev Bras Hematol Hemoter. 2017;39(1):20–7.
65. Sam C, Desai P, Laber D, Patel A, Visweshwar N, Jaglal M. Pegylated bovine carboxyhaemoglobin utilisation in a thrombotic thrombocytopenic purpura patient. Transfus Med. 2017;27(4):300–2.
66. McConachie S, Wahby K, Almadrahi Z, Wilhelm S. Early experiences with PEGylated carboxyhemoglobin bovine in anemic Jehovah's witnesses: a case series and review of the literature. J Pharm Pract. 2020;33(3):372–7.
67. Thenuwara K, Thomas J, Ibsen M, Ituk U, Choi K, Nickel E, et al. Use of hyperbaric oxygen therapy and PEGylated carboxyhemoglobin bovine in a Jehovah's witness with life-threatening anemia following postpartum hemorrhage. Int J Obstet Anesth. 2017;29:73–80.
68. Holzner ML, DeMaria S, Haydel B, Smith N, Flaherty D, Florman S. Pegylated bovine carboxyhemoglobin (SANGUINATE) in a Jehovah's witness undergoing liver transplant: a case report. Transplant Proc. 2018;50(10):4012–4.
69. Brotman I, Kocher M, McHugh S. Bovine hemoglobin-based oxygen carrier treatment in a severely anemic Jehovah's witness patient after cystoprostatectomy and nephrectomy: a case report. A A Pract. 2019;12(7):243–5.
70. Alayash AI. Mechanisms of toxicity and modulation of hemoglobin-based oxygen carriers. Shock. 2019;52(1S Suppl 1):41–9.
71. Silverman Toby A, Weiskopf Richard B. For the planning C, the S. hemoglobin-based oxygen carriers: current status and future directions. Anesthesiology. 2009;111(5):946–63.
72. Estep TN. Haemoglobin-based oxygen carriers and myocardial infarction. Artif Cell Nanomed Biotechnol. 2019;47(1):593–601.
73. Hare GM, Hum KM, Kim SY, Barr A, Baker AJ, Mazer CD. Increased cerebral tissue oxygen tension after extensive hemodilution with a hemoglobin-based oxygen carrier. Anesth Analg. 2004;99(2):528–35, table of contents.
74. Korte EA, Pozzi N, Wardrip N, Ayyoubi MT, Jortani SA. Analytical interference of HBOC-201 (Hemopure, a synthetic hemoglobin-based oxygen carrier) on four common clinical chemistry platforms. Clin Chim Acta. 2018;482:33–9.

On the Oxidative Toxicity of Hemoglobin

14

Abdu I. Alayash

Red Blood Cell Oxidation Reactions in Health and Disease: What Have We Learned?

Oxidation of Hemoglobin and Cytosolic Antioxidative Proteins

The heme prosthetic group in the ferrous Hb ($HbFe^{2+}$) reversibly binds oxygen as the RBC cycles from the lungs to the tissues and back again. Over time this process leads to oxidation of the heme iron to the ferric form ($HbFe^{3+}$), a process known as autoxidation (akin to iron rusting in non-biological systems). The degree of the spontaneous oxidation of Hb may reach up to 1–3% of the total Hb in healthy RBCs despite the presence of several antioxidant proteins that maintain the iron oxidation at a tolerable level under normal physiological conditions [1].

The slow autoxidation of the oxygen-carrying $HbFe^{2+} \rightarrow HbFe^{3+}$, a non-oxygen carrying methemoglobin (metHb), is a single electron process which leads to the production of superoxide ions ($O2^{\bullet -}$) that rapidly dismutate to form hydrogen peroxide (H_2O_2), a powerful oxidant (Eq. (14.1)). The resulting H_2O_2 triggers a cascade of oxidative reactions with either ferrous (Eq. 14.2) or ferric Hb (Eq. 14.3). These reactions lead to the generation of the potent oxidizing ferrylHb ($HbFe^{4+}$) species, as well as a secondary radical from the reaction between H_2O_2 and metHb. However, unlike classical peroxidases, Hb is unable to harness this radical which then migrates to a group of amino acids in an area on the protein known as the oxidation "hotspot", primarily located at the βCys93 site [2].

$$HbFe^{2+}O_2 \rightarrow HbFe^{3+} + O_2^{\bullet -} \text{Autoxidation} \quad (14.1)$$

$$HbFe^{2+}O_2 + H_2O_2 \rightarrow HbFe^{4+} + O_2^{-} + H_2O + O_2 \text{Oxidative Modifications} \quad (14.2)$$

$$HbFe^{3+} + H_2O_2 \rightarrow {}^{\bullet}HbFe^{4+} + O_2^{-} + H_2O \text{ Radical Migration to } \beta\text{Cys93} \quad (14.3)$$

In addition to the spontaneous process of Hb iron oxidation, RBCs are continuously exposed to both endogenous and exogenous sources of reactive oxygen species (ROS) that can impact the RBC's flexibility and deformability [3].

RBCs maintain powerful antioxidative machinery primarily designed to keep Hb in the ferrous functional form during circulation. When these cells reach the end of their life span, oxidation of Hb and oxidative side reactions appear to intensify due to the weakening of antioxidative defense mechanisms [3]. RBCs antioxidant defense enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GR), in addition to low-molecular-weight antioxidants, such as glutathione (GSH), and vitamins E and C. Furthermore, RBCs have a plasma membrane redox system that transfers electrons from intracellular substrates to extracellular electron acceptors, which may be NAD+ or vitamin C. Therefore, RBCs uniquely function to protect Hb via a selective barrier allowing gaseous and other ligand transport as well as providing enzymatic mechanisms to maintain Hb in a functional nontoxic state [4].

The Role of Red Cell Band 3 in Hemoglobin Oxidation

Band 3 and its associated proteins are an integral part of the RBC membrane that function primarily in Cl^- and $HCO3^-$ exchange, thus maintaining acid balance, ion distribution across the RBC membrane, and cell shape integrity and durability [5]. In recent years, band 3's role in the overall mainte-

*This book chapter reflects the views of the author and should not be construed to represent FDA's views or policies.

A. I. Alayash (✉)
Laboratory of Biochemistry and Vascular Biology, Center for Biologics Evaluation and Research, Food and Drug Administration (FDA), Silver Spring, MD, USA
e-mail: abdu.alayash@fda.hhs.gov

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_14

nance of redox balance in RBCs has been recognized. Consequently, due to band 3's ion exchange capability, it has served as a suitable cell model to evaluate RBCs response to oxidative conditions [6].

The Hb-conformation–dependent interaction with band 3 proteins and its involvement in regulating glycolysis is well documented [7], but the interplay between Hb-mediated oxidation reactions (e.g., βCys93 oxidation) and band 3 is less defined. However, recent research on blood from normal, transgenic sickle cell disease (SCD) mice and from patients with SCD showed that there was indeed a direct contact between Hb oxidative intermediates specifically ferryl Hb (but not hemichromes) with band 3 [8, 9]. Using a proteomic methodology to analyze RBCs from sickle cell disease (SCD) mice and patients, we showed that the redox transition of Hb into a higher oxidation state (ferryl) interacts directly with band 3 resulting into oxidative modifications of band 3 network of proteins and the appearance of other PTMs such phosphorylation and ubiquitination of Hb itself [9, 10] (see below).

Hemoglobin-Driven Oxidation Reactions Promote Microparticles Formation

SCD is a genetic disorder due primarily to a single point mutation in the β-globin gene resulting into a non-charged amino acid, Val replacing a negatively charged Glu at the position 6. In the deoxyHb conformation, a "sticky patch" is formed between Val and other close by non-charged amino acids resulting in the polymerization of deoxyHb molecules into long fibers within RBCs. The transformation of RBCs into less deformable and flexible carrier as it navigates narrow microcapillaries lead to vaso-occlusion and ultimately hemolysis [1]. Sickle cell RBCs are continuously subjected to endogenous and exogenous oxidative stresses. Among the recently discovered sources of ROS was the finding that these cells maintain an active form of NADH oxidases [11]. Another recent finding reported that SCD RBCs retain some mitochondria that can also be a source of reactive oxygen species (ROS) [12].

Recent mechanistic studies confirmed that oxidative stress within SCD RBCs are driven by Hb oxidation side reactions [9, 10]. However, the potential physiological ramifications of these reactions are not yet fully characterized. Separately from the unusual differences in oxidative milieu within SCD RBCs vs. normal ones, the isolated HbS has been shown to be more oxidatively unstable than HbA, possessing profound differences in enzymatic activities as represented by the pseudoperoxidative reactions described in Eqs. 14.1, 14.2, and 14.3. Isolated HbS has been shown to autoxidize at rates several fold faster than normal Hb. For example, in the presence of a bolus amount of peroxide, ferryl Hb is rapidly formed. However, unlike HbA ferrylHbS persists longer in solutions and autoreduced at slower rates than HbA. This unusual redox cycle results in oxidative modifications and misfolding or unfolding of ferrylHbS which ultimately leads to the toxic loss of heme [13, 14].

We have documented that Hb transformation to higher oxidative forms is markedly increased in microparticles (MPs) generated from SCD mice and human SCD samples, although these Hb oxidative forms were also detected in non-microparticle enriched RBC specimens as well. Ferryl Hb (once formed), manifests in several oxidative changes, including irreversible oxidation of βCys93 and ubiquitination of βLys96 and βLys145. Ferryl Hb also oxidatively targeted band 3, which resulted in a band 3–mediated complex formation (with Hb and other membrane proteins) and activation of the ubiquitin–proteasome system (UPS) pathway. When MPs were incubated with endothelial cells, this led to mitochondrial dysfunction and apoptotic cell death. These mechanistic results suggest that Hb oxidative pathways drive oxidative changes within SCD RBCs, and similarly, in RBC-derived MPs, causing us to hypothesize that antioxidative interventions may slow down and/or prevent Hb-oxidative side reactions in SCD [9, 10].

Role of β Cysteine 93 in Oxidative Stability of Hemoglobin

Because these oxidation reactions (described above) precede at faster rates in HbS than HbA solutions, we were able to examine the structural and other stability governing parameters more closely in the HbS protein. Using a variety of photometric, mass spectrometric, and electron paramagnetic resonance (EPR) techniques we were able to define the oxidative pathways within the Hb molecule and identify target(s) of these reactions outside of the molecule. We found that because HbS is unable to harness the ferryl radicals effectively, this radical migrates to what is known as the "oxidation hotspot" which includes a cluster of susceptible amino acids and irreversibly oxidizes the β cysteine 93 residue [13, 14].

In addition to its well-established role in allosteric mechanisms, βCys93 is involved in the transport of nitric oxide (NO), and detoxification of $O2^{-}$ [2]. Several studies from our laboratory and others have shown that βCys93 (positioned on the surface near of an F helix located at the β = β subunit interface) is an endpoint for free radical induced Hb oxidation which occurs due to oxygen binding and concomitant H_2O_2 (and $O2^{-}$) production [15]. βCys93 is readily and irreversibly oxidized in the presence of a mild oxidant, H_2O_2, to cysteic acid, which leads to destabilization of Hb, improper

protein folding, and the loss of heme. Oxidized βCys93 is therefore a useful reporter on the oxidative status of Hb in RBCs intended for transfusion or within RBCs from patients with hemoglobinopathies. Accordingly, site specific mutation of a redox active amino acid (s) to reduce the ferryl heme, or direct chemical modifications that can shield βCys93 have been proposed to improve oxidative resistance of Hb, and may offer a protective therapeutic strategy [2, 16].

Genetically and Chemically Modified HBOCs: The Impact of Molecular Modifications

Effects of Chemical and Genetic Modifications on the Oxidation of HBOCs

Generally, all HBOCs are derived from outdated human blood or in some cases from animal blood. Hb is isolated from RBCs and is then purified to remove RBC's proteins using conventional separation techniques and filtration methods. Some manufactures employ further chromatographic methods to produce a highly purified HbA (known as HbA_0) as starting material for subsequent chemical modifications [17]. Others, however, used what is known as stroma free (poor) Hb (SFH), which in theory contains other RBC proteins [18]. Two major chemical modifications have been widely used to generate Hbs, specifically glutaraldehyde and polyethylene glycol which introduce inter and intramolecular modifications, producing stabilized tetramers or conjugated/polymerized molecules. In many cases, these chemical reagents used in the modifications of Hb resulted in non-site-specific and random modifications of the Hb molecules [19].

Alternatively, genetic modifications of Hb have also been utilized as HBOCs. One manufacturer genetically engineered tetramer of Hb in *E Coli* based on the mutant Presbyterian Hb, where two alpha subunits were covalently joined by adding a single amino acid (Gly) and was advanced to early clinical trials [20]. Other variations of this model system were also were produced by academic institutions [21]. Encapsulating Hb in a vesicle was also investigated as model system for artificial RBCs containing enzymes and other essential proteins. These vesicles did not initially proceed beyond preclinical animal studies, although a second-generation version of these vesicles are now under investigation [22]. Generally, HBOCs were found to be more susceptible to autoxidation, oxidative changes and heme loss, in large part due to the random nature of chemical modifications occurring outside of RBCs [23]. Under oxidative stress conditions, human, bovine, or recombinant HBOCs undergo, oxidative changes that were more exaggerated leading to the accumulation of higher levels of ferryl compared to the unmodified human or bovine forms of these proteins [23].

Effects of Altering Oxygen Affinity of HBOCs on Autoxidation and Oxidative Changes

In a landmark paper [23], we have recently completed a comprehensive biochemical and biophysical comparison of all human, bovine and genetically engineered HBOCs that have (at least in some cases) been tested in humans in late clinical trials. Some of these commercial HBOCs were only recently made available to independent investigators. Toward this goal, oxygen equilibrium and ligand binding kinetics under different experimental conditions as well as their oxidation kinetics, redox reactions, and heme release kinetics were determined for all HBOCs. Generally, polymerization of the proteins with glutaraldehyde and PEGylation with POE induced changes in the position and shapes of the oxygen equilibrium curves (OECs) when compared to the oxygen affinity of native human and bovine Hb molecules. A common phenomenon among some polymerized HBOCs was the lack of saturation of their OECs at the normal physiological range of 90–100 mmHg. However, using complete OECs generated by the Adair constants allowed us to accurately determine oxygen binding parameters for these modified Hbs to reflect their true oxygen binding properties under physiological conditions. Another consequence of chemical modifications was the loss of oxygen binding cooperativity reflected by the reduction in the value of the Hill coefficients (n) calculated for some of these HBOCs when contrasted to values of the native human and bovine proteins respectively. The Bohr effect curves for oxygen binding to human and bovine HBOCs along with those obtained for the native proteins under the same pH range were also determined. A classical sigmoidal Bohr effect curve can be seen with native human and bovine Hbs over a wide range of acidic and alkaline pHs, but a pronounced reduction in the Bohr effects for almost all HBOCs was observed [23]. This reduction in HBOC Bohr effects over varying pHs suggests these modified forms of extracellular Hb may not be as robust to normal fluctuations in pH that occur in blood. In this study, almost all known HBOCs with their diverse oxygen affinities, showed no strong correlation between heme iron autoxidation rates and their respective oxygen affinities (P_{50}) [24]. This potentially creates opportunities for designing HBOCs with low oxygen affinity without compromising their autoxidative side reactions.

Effects of Chemical Modifications on Heme Loss From HBOCs

Early strategies designed to address rapid clearance of HBOCs associated with tetramer dissociation into dimers, included chemical, physical, or genetic methods, such as

polymerization, conjugation, and encapsulation into vesicles were relatively successful [25].

Polymerization and conjugation strategies significantly mitigated problems associated with HOBC's short circulation life span and renal toxicity. However, autoxidation and oxidative side reactions produces structural changes and unfolding of the HBOCs despite the introduction of inter and intra-molecular crosslinking modifications [26].

Using a recombinant holomyglobin (with no heme) as a receptor, heme was extracted from all known HBOCs (including ferric forms). The rates of heme loss from all HBOCs were recently reported. In these experiments, the rates for heme transfer were recorded after rapid mixing at room temperature. HBOCs showed different rates for heme loss, with uncross-linked HBOCs exhibiting the highest rates of heme loss. Additionally, another source of heme loss was due to HBOC dimerization, monitored by reacting HBOCs with haptoglobin (Hp), since Hp rapidly reacts with Hb dimers [23].

Effects of Non-site-Specific modifications on Oxidative Stability of HBOCs

Polymerizing reagents such as glutaraldehyde are known to randomly modify the Hb molecule at the inter and intramolecular sites. This non-site-specific modification of the protein generates a wide range of molecular isomers with a wide range of molecular weight distributions. This form of chemical modifications is widely used in polymerizing human and bovine SFHs resulting in changes in oxygen binding characteristics and autoxidation kinetics [23]. PEGygalation of Hb with polyethylene glycol-based chemistries is also highly non-site-specific although several groups have introduced small linkers targeting Cys93 and other amino acids to site specifically attach the PEG molecule(s) [27]. Intra-and intermolecularly cross-linked human HbA_0 with activated sugar, *O*-raffinose, is an example of how non-site-specific modification of the protein impacts its stability and safety which is described below [28, 29].

Reacting human deoxyHbA_0 with oxidized raffinose (*O*-raffinose), a trisaccharide, results in a low oxygen affinity "blood substitute," stabilized in a noncooperative T-conformation. We extensively characterized this Hb by first fractionating the *O*-raffinose-modified HbA_0 heterogeneous polymer (*O*-R-PolyHbA_0) into six distinct fractions with a molecular weight distribution ranging from 64 to approximately 600 kDa using size-exclusion chromatography (SEC). Oxygen equilibrium and kinetics binding parameters of all fractions were nearly identical, reflecting a lack of heterogeneity in ligand binding properties among all the *O*-R-PolyHbA_0 fractions (Hill coefficient (*n*) equals to 1.0). We used several mass spectrometry techniques to investigate undigested and digested HbA_0, intact *O*-R-PolyHbA_0, and *O*-R-PolyHbA_0 fractions. To our surprise the proposed sites of intramolecular crosslinking (i.e., β1Lys82, β2Lys82, and β1Val1) were not found to be the predominant site of crosslinking within the central cavity as claimed by the manufacture of this Hb [30]. Intermolecular crosslinking with *O*-raffinose results in no discernible modification sites except for βCys93 and αCys104 leading accelerated iron oxidation. This random non-site-specific modification of βCys93 which is known to reflect oxidative stability of the protein and may have provided some basis for the reported toxicity of this oxygen carrier [30].

Countermeasures Against Hemoglobin Oxidation and the Design of Safer HBOCs

Intercepting Nitric Oxide and the Control of Hemodynamic Imbalance

In early 1980, NO was discovered to autoregulate systemic and pulmonary vascular tone (see recent review [31]). If NO, a dilator of blood vessels, is removed by infused HBOCs, this would result in vasoconstriction of blood vessels, systemic and pulmonary hypertension, and decreased cardiac output. The hemodynamic imbalance caused by removal of NO though is transient in nature (1–2 hours duration of blood pressure elevation). This simple reaction however, impacted the field of HBOCs dramatically and controlling vasoactivity associated with HBOCs had become a priority research and developmental goal [32]. The reaction is primarily with the heme group, which can be completed within a few seconds with a profound consequence such as blood vessel constriction and elevation in systematic and pulmonary blood pressures (approximate mean arterial blood pressure changes ranges between15–30 mmHg). However, blood pressure elevations seen in animals and humans appear to follow a predictable path that can return to normal within two hours [33].

In 1996 several intriguing hypotheses were put forward suggesting in some cases that Hb within RBCs is capable of transporting and exporting NO to βCys93 through an allosteric transition between the relaxed (R) and tense (T) states of the Hb molecule. This so called "SNO-Hb" is ultimately exported by the RBCs to effect blood vessel vasodilation [34]. An alternative hypothesis suggested however, that nitrite can enzymatically transform Hb to become a source for NO (e.g., nitrite reductase) that requires deoxyHb as a starting intermediate [35]. These two hypotheses were based on in vitro experiments with little or no validated animal studies to support them. Exporting NO from the RBC or

from free Hb or pharmacologically inducing Hb reductase ability to generate NO would be mechanistically difficult without intermediates, particularly since Hb rapidly and irreversibly consumes NO. In a rush to capitalize on this newly founded property of NO, patients SCD were given Sildenafil citrate to facilitate NO production, which resulted in unexpected outcome and termination of this trial (https://www.nlm.nih.gov/databases/alerts/pulmonary_hypertension.html). In other studies, in which large animals (swine) experiencing hemorrhagic shock to mimic battlefield conditions, nitrite infusion with an HBOC resulted in a transient drop in blood pressure and increased the risk of pulmonary complications including pulmonary edema as well as congestion [36].

Several approaches designed to overcome the Hb-NO reaction include vesicle encapsulation in order to maintain Hb within a 'protected' space, as a barrier to detrimental vasoactive mechanisms. Other approaches have included adding anti-hypertensive agents to block specific areas of vascular control, that are potentially involved in the interaction between Hb and the vascular system. Research efforts are also focused on controlling elevated blood pressure after infusion of HBOCs using NO donors, or the inhibition of NO synthetic pathways, but unfortunately this had little or no long-term effects on organ toxicity [26].

Antioxidants and Reducing Agents for the Control HBOC Oxidative Toxicity

Several ex vivo and in vivo studies have demonstrated a critical role for plasma antioxidants such as ascorbic acid (AA) and uric acid (UA) in maintaining Hb and HBOCs in functional non-oxidized states [26]. These two naturally occurring antioxidants are critical for maintaining HBOCs in the reduced and functional states. This is particularly important for the maintenance of the ferrous form of Hb, which is necessary for effective oxygen delivery. The oxidation to ferric Hb triggers other steps in the oxidative pathway which increase the toxicity of Hb (Eqs. 14.1, 14.2, and 14.3).

Humans and non-human mammal such as guinea pigs are unable to synthesize endogenous AA due to an evolutionary loss of hepatic L-gluconolactone oxidase (LGO) gene expression, a key enzyme in AA biosynthetic pathways [37]. The antioxidant activity of critical enzymes such as superoxide dismutase (SOD) in tissues from non-AA producing species is significantly elevated compared to AA producing species (e.g. rats and mice). Moreover, the role of other plasma antioxidants, particularly UA has evolved in the absence of AA production [38].

We have previously demonstrated the power of endogenous antioxidant mechanisms by comparing the toxicokinetics of an HBOC (polymerized bovine Hb; Oxyglobin™) administered to a rat (AA producer) versus the administration of the same HBOC to a guinea pig (non-AA producer). This experiment demonstrated that ferric HBOC levels were 4-fold greater in the guinea pig compared to the rat, and that oxidative instability of the HBOC became evident following administration in the guinea pig but not the rat [39]. Using HPLC and mass spectrometric methods to analyze blood samples from these animals confirmed oxidative changes of circulating HBOCs in non-AA-producing animals versus those with AA [39].

More recently HBOC developers became more amenable to the use of antioxidants such as AA, as an additive to HBOC solutions to control its oxidation in human circulation. A recent case of a Jehovah Witness patient who lost a considerable amount blood that was given large doses of AA. HBOC oxidation was considerably reduced after reaching as high as 50% of total Hb infused in this patient [40].

Other antioxidants such sodium selenite reduced Hb-induced epithelial damage to intestinal mucosa and Hb-induced venular leakage in the rat mesentery [41] Acetylcysteine is another antioxidant that has been co-administered with several HBOCs in vivo [42]. Overall, the co-administration of certain antioxidants with HBOCs may aid in the attenuation of oxidative reactions.

RBC antioxidative enzymes (primarily SOD and catalase) that maintain Hb in the ferrous functional form and that increase in response to ROS-induced oxidative stress have been crosslinked to the Hb molecule in an attempt to control Hb's own ROS [43]. For example, glutathione (GSH) is an important scavenger of free radicals and a potent endogenous antioxidant, which helps to protect RBCs from oxidative injury has also been crosslinked to Hb [44].

Engineering Oxidatively Stable HBOCs Based on Mutant Hemoglobins: The Case of the Providence Mutation

There are thousands of naturally occurring variant forms of Hb in all populations in diverse geographical areas worldwide. Due to evolutionary pressures, these Hb variants are found in some cases to resist oxidative challenges. However, most of these mutations enhance oxidative degradation and ultimately result in circulatory disorders [45].

We previously discovered that a naturally occurring mutant Hb Providence (βLys82 → Asp) (βK82D), was much more resistant to degradation by H_2O_2 than normal human HbA [46]. Based on this initial finding, we subsequently engineered this mutation into a genetically cross-linked Hb tetramer (rHb0.1/βK82D) and demonstrated that the βK82D mutation conferred more resistance to degradation by H_2O_2

via markedly inhibiting oxidation of the β93 cysteine side chain [47]. Next, we tested this extraordinary stability of the βK82D mutation in another Hb model system known for its oxidative instability, e.g., sickle cell Hb (HbS). The HbS (βE6V) mutation is known to oxidize faster than normal HbA and remains longer in a highly oxidizing ferryl form ($HbFe^{4+}$), which then targets the "hotspot" amino acids including βCys93 [13]. We found that the (βE6V/βK82D) form of Hb added significant oxidative resistance to βE6V when challenged with H_2O_2, in addition to a dramatic improvement in the delay times and polymerization of βE6V [14].

The basis of this extraordinary protection afforded by βK82D in both sickle cell and more so in the crosslinked Hb lies within the fact that this substitution may minimize the levels of irreversible oxidation of βCys93. Oxidation of βCys93 to cysteic acid is known to perturb the extensive network of hydrogen bonding and salt bridges at the interface between the β2 FG corner. The substitution of the native βLys82 for Asp82, (located ~18.3 Å away from the β-heme) is not known to be engaged in electron transfer with the heme [47]. However, the higher oxidative stability caused by the βK82D mutation has been attributed to changes in reactivity of the βCys93 side chain, which may be due to either indirect electrostatic effects or alterations to the local dynamics of the protein structure [14].

We recently investigated the impact of the βK82D mutation on tissue oxygenation and mitochondrial function. The rationale for this is based on anecdotal reports on one patient with the βK82D mutation exhibiting mild anemia and erythropoiesis attributed to alterations in the oxygen binding properties of the patients' blood. Specifically, this patient had a higher P_{50} than normal subjects (i.e., P_{50} = 21.1 mm Hg vs, control P_{50} = 29.0 mm Hg) [48]. Additionally, there was also another rare case of an SCD patient with a βK82D mutation who had attenuated disease symptoms [49]. In the context of SCD, the impact of the βK82D mutation on the course and the severity of the disease is perhaps not entirely due to a small left shift in the oxygen equilibrium curve (smaller P50), but rather may be due to an overall improvement in patient's resistance to oxidative stress [47].

Exploring the impact of introducing βK82D containing Hb on vascular endothelial redox homeostasis and energy metabolism in cultured endothelial cells led to the finding of altered bioenergetic function via enhanced basal cellular glycolysis and glycolytic capacity. Additionally, the treatment of cells with Hb variants containing βK82D also resulted in lower heme oxygenase-1 and ferritin expressions compared to native Hbs. It appears that that the presence of βK82D confers significant resistance to oxidative toxicity. In conclusion, the Providence βK82D mutation provides a potential gene-editing target for the treatment of sickle cell disease and for safe and effective oxygen therapeutics [16].

Haptoglobin and hemopexin Are First-Line-of Defense against Free Hemoglobin and Heme

During hemolysis, Hp binds tightly to Hb subunits ($\alpha\beta$ dimers) in the circulation. This Hb-Hp complex is then chaperoned to macrophages for safe degradation through the CD163 receptor on the macrophage membrane and heme is subsequently degraded into carbon monoxide (CO), bilirubin, and biliverdin byproducts [50]. Structurally, Hp is a tetramer consisting of two α-chains (each approximately 9 kDa) and two β-chains (each approximately 33 kDa) which are covalently linked by disulfide bonds. The binding between Hp and Hb is among the strongest non- covalent interactions known in biological systems. When released in plasma, the Hb–Hp complex impairs filtration and clearance of Hb dimers by the kidney, and instead directs Hb to CD163 on to macrophages for a process of endocytosis and final degradation (for review, see [50]. Besides physical sequestration of Hb through this strong binding, Hp also detoxifies Hb and restricts Hb's peroxidative side reactions known to be toxic to tissues.

In recent years, we have investigated the mechanism afforded by Hp to Hb at the molecular level. The Hp molecule provides protection for Hb against oxidants by binding close to the so-called hotspot amino acids (of Hb's hotspots amino acids including βCys93) revealed recently in crystallography studies [51]. Secondly, Hp has been shown to allow the heme active site to operate unhindered, leading to the elimination of oxidants and diffusion of radicals emanating from the heme to key Tyr residues on the Hb molecule [52, 53]. This radical reactivity may ultimately be directed to the Hp molecule resulting in a safer redox inactive state for the Hb molecule [54]. Another advantage of complexing Hb and Hp is that this newly formed complex retards heme release for a considerable amount of time [54].

There remains an unanswered question as to whether Hp can be clinically useful in clearing and oxidatively inactivating HBOCs that are generally stabilized in tetrameric and polymeric forms. Particularly, since Hp may primarily bind to dimeric Hb as rapid mixing kinetics of Hp with a number of intra- and intramolecularly crosslinked HBOCs showed little or no binding, whereas uncrosslinked conjugated HBOCs bound very strongly [23]. We also surprisingly found that β-crosslinked HBOCs in a tetrameric form had a strong affinity for Hp than α-crosslinked proteins [55]. Recent animal studies have confirmed that Hb compartmentalization (rather than short-lived NO-based therapies) with molecules having Hp behavior may be useful in countering vasoactive and oxidative toxicities associated with free Hb in hemolytic anemias [56, 57].

Hemopexin (HPX) is an acute phase plasma protein, found in the circulation at concentrations ranging from 8 to 21 mM [58]. HPX serves as a specific

scavenger of plasma heme and clearance by transporting it to the liver, and like Hp, HPX functions as a key plasma protector against Hb oxidation by-products. When compared with other plasma heme scavengers (such as albumin, high- and low-density lipoproteins, and *a*1-micro- globin), HPX has the tightest binding kinetics [59]. In numerous in vivo studies in dogs, guinea pigs, and sickle cell mice, Hp and HPX have been shown to limit the toxic effects of infused cell-free Hb [56, 60].

Summary and Conclusion

Despite the active research and developmental efforts over the last 3 decades to design safe and effective HBOCs, no viable candidate has been approved for clinical use in the United States. Much effort by industry and academia was invested in resolving vasoactivity common to all HBOCs, while oxidative toxicity triggered by these products was not fully appreciated, nor fully investigated. A better understating of the molecular genesis of Hb oxidative toxicity has led in recent years to the advancement of new molecular approaches and interventions that will be more likely to succeed as HBOCs that have an acceptable safety profile that are ready for clinical use (see reference [19] for more details).

Key Points

- Recent research reveals that hemoglobin drives internal cytosolic and membrane oxidative changes within red blood cells.
- Similar, but more exaggerated oxidative side reactions occur when hemoglobin is free due to hemolysis or when hemoglobin is used as an HBOC.
- Molecular intervention designs are now in place to control hemoglobin's internal oxidative changes together with the inclusion of antioxidant additives may provide a sufficiently safe HBOC therapeutic product.

References

1. Dickerson RE, Gies I. Hemoglobin: structure, function, evolution, and pathology. California: The Benjamin/Cummings Publishing Company; 1983.
2. Alayash AI. βCysteine 93 in human hemoglobin: a gateway to oxidative stability in health and disease. Lab Invest. 2020. https://doi.org/10.1038/s41374-020-00492-3.
3. Nagababu E, Chrest FJ, Rifkind JM. Hydrogen-peroxide-induced heme degradation in red blood cells: the protective roles of catalase and glutathione peroxidase. Biochim Biophys Acta. 2003;1620:211–7.
4. Rizvi SI, Jha R, Maurya PK. Erythrocyte plasma membrane redox system in human aging. Rejuvenation Res. 2006;9:470–4.
5. Jay DG. Role of band 3 in homeostasis and cell shape. Cell. 1996;86:853–4. https://doi.org/10.1016/s0092-8674(00)80160-9.
6. Morabito R, Remigante A, Cordaro M, Trichilo V, Loddo S, Dossena S, Marino A. Impact of acute inflammation on band 3 protein anion exchange capability in human erythrocytes. Arch Physiol Biochem. 2020:1–7. https://doi.org/10.1080/13813455.2020.1764048 36.
7. Rogers SC, et al. Sickle hemoglobin disturbs normal coupling among erythrocyte O2 content, glycolysis, and antioxidant capacity. Blood. 2013;121(9):1651–62. https://doi.org/10.1182/blood-2012-02-414037.
8. Welbourn EM, Wilson MT, Yusof A, Metodiev MV, Cooper CE. The mechanism of formation, structure and physiological relevance of covalent hemoglobin attachment to the erythrocyte membrane. Free Radic Biol Med. 2017;103:95–106.
9. Jana S, Strader MB, Meng F, Hicks W, Kassa T, Tarandovskiy I, De Paoli S, Simak J, Heaven MR, Belcher JD, Vercellotti GM, Alayash AI. JCI Insight. 2018;3(21):e120451. https://doi.org/10.1172/jci.insight.120451. PMID: 30385713.
10. Strader MB, Jana S, Meng F, Heaven MR, Shet AS, Thein SL. Alayash AI post-translational modification as a response to cellular stress induced by hemoglobin oxidation in sickle cell disease. Sci Rep. 2020;10(1):14218. https://doi.org/10.1038/s41598-020-71096-6. PMID: 32848178.
11. George A, et al. Erythrocyte NADPH oxidase activity modulated by Rac GTPases, PKC, and plasma cytokines contributes to oxidative stress in sickle cell disease. Blood. 2013;121:2099–107. https://doi.org/10.1182/blood-2012-07-441188.
12. Jagadeeswaran R, et al. Pharmacological inhibition of LSD1 and mTOR reduces mitochondrial retention and associated ROS levels in the red blood cells of sickle cell disease. Exp Hematol. 2017;50:46–52. https://doi.org/10.1016/j.exphem.2017.02.003.
13. Kassa T, et al. Sickle cell hemoglobin in the ferryl state promotes betaCys-93 oxidation and mitochondrial dysfunction in epithelial lung cells (E10). J Biol Chem. 2015;290:27939–58. https://doi.org/10.1074/jbc.M115.651257.
14. Meng F, Kassa T, Strader MB, Soman J, Olson JS, Alayash AI. Substitutions in the β subunits of sickle-cell hemoglobin improve oxidative stability and increase the delay time of sickle-cell fiber formation. J Biol Chem. 2019;294:4145–59.
15. Jia Y, Buehler PW, Boykins RA, Venable RM, Alayash AI. Structural basis of peroxide-mediated changes in human hemoglobin: a novel oxidative pathway. J Biol Chem. 2007;282:4894–907.
16. Jana S, Strader MB, Alayash AI. The Providence mutation (βK82D) in human hemoglobin substantially reduces βcysteine 93 oxidation and oxidative stress in endothelial cells. Int J Mol Sci. 2020;21(24):E9453. https://doi.org/10.3390/ijms21249453. PMID: 33322551 C.
17. Adamson JG, Moore C. Hemolink, an O-Raffinose crosslinked hemoglobin-based oxygen carrier. In: Chang TMS, editor. In blood substitutes, principles, methods, products and clinical trials; 1998. pp. 62–79.
18. Privalle T, Talarico T, Keng T, et al. Pyridoxalated hemoglobin polyoxyethylene: a nitric oxide scavenger with antioxidant activity for the treatment of nitric oxide-induced shock. Free Radic Biol Med. 2000;28:1507–17.
19. Alayash AI. Mechanisms of toxicity and modulation of hemoglobin-based oxygen carriers. Shock. 2019;(1S Suppl 1):41–49. https://doi.org/10.1097/SHK. PMID: 29112106.
20. Looker D, Abbott-Brown D, Cozart P, Durfee S, Hoffman S, et al. A human recombinant haemoglobin designed for use as a blood substitute. Nature. 1992;356:258–60.
21. Alomari E, Ronda L, Bruno S, Paredi G, Marchetti M, Bettati S, et al. High- and low-affinity PEGylated hemoglobin-based oxygen carriers: differential oxidative stress in a Guinea pig transfu-

sion model. Free Radic Biol Med. 2018;124:299–310. https://doi.org/10.1016/j.freeradbiomed.2018.06.018.
22. Azuma H, Fujihara M, Sakai H. Biocompatibility of HbV: liposome-encapsulated hemoglobin molecules-liposome effects on immune function. J Funct Biomater. 2017;8(3):24. https://doi.org/10.3390/jfb8030024.
23. Meng F, Kassa T, Jana S, Wood F, Zhang X, Jia Y, et al. Comprehensive biochemical and biophysical characterization of hemoglobin-based oxygen carrier therapeutics: all HBOCs are not created equally. Bioconjug Chem. 2018;29:1560–75.
24. Nagababu E, Ramasamy S, Rifkind JM, Jia Y, Alayash AI. Site-specific cross-linking of human and bovine hemoglobins differentially alters oxygen binding and redox side reactions producing rhombic heme and heme degradation. Biochemistry. 2002;41:7407–15. https://doi.org/10.1021/bi0121048.
25. Taguchi K, Yamasaki K, Maruyama T, Otagiri M. Comparison of the pharmacokinetic properties of hemoglobin-based oxygen carriers. J Funct Biomater. 2017. https://doi.org/10.3390/jfb8010011.
26. Buehler PW, Alayash AI. Oxidation of hemoglobin: mechanisms of control in vitro and in vivo. Transfus Altern Transfus Med. 2007;9:204–12.
27. Khan I, Dantsker D, Samuni U, Friedman AJ, Bonaventura C, Manjula B, et al. βCys393 modified hemoglobin: kinetic and conformational consequences. Biochemistry. 2001;40:7581–92.
28. Hill SE, Gottschalk LI, Grichnik K. Safety and preliminary efficacy of hemoglobin raffimer for patients undergoing coronary artery bypass surgery. J Cardiothorac Vasc Anesth 2002. https://doi.org/10.1053/jcan.2002.128416.
29. Silverman TA, Weiskopf RB, Planning Committee and the Speakers. Hemoglobin-based oxygen carriers: current status and future directions. Anesthesiology. 2009; https://doi.org/10.1097/ALN.0b013e3181ba3c2c.
30. Boykins RA, Buehler PW, Jia Y, Venable R, Alayash AI. O-raffinose crosslinked hemoglobin lacks site-specific chemistry in the central cavity: structural and functional consequences of beta93Cys modification. Proteins. 2005. https://doi.org/10.1002/prot.20453.
31. Lancaster JR Jr. Historical origins of the discovery of mammalian nitric oxide (nitrogen monoxide) production/physiology/pathophysiology. Biochem Pharmacol. 2020. https://doi.org/10.1016/j.bcp.2020.113793.
32. Estep TN. Issues in the development of hemoglobin-based oxygen carriers. Semin Hematol. 2019; https://doi.org/10.1053/j.seminhematol.2019.11.006.
33. Yu B, Shahid M, Egorina EM, Sovershaev MA, Raher MJ, Lei C, et al. Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobin-based oxygen carrier. Anesthesiology. 2010. https://doi.org/10.1097/ALN.0b013e3181cd7838.
34. Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. Nature. 1996;380:221–6.
35. Minneci PC, Deans KJ, Shiva S, Zhi H, Banks SM, Kern S, et al. Nitrite reductase activity of hemoglobin as a systemic nitric oxide generator mechanism to detoxify plasma hemoglobin produced during hemolysis. Am J Physiol. 2008;295:H743–54.
36. Moon-Massat P, Scultetus A, Arnaud F, Brown A, Haque A, Saha B, et al. Effect of HBOC-201 and sodium nitrite resuscitation after uncontrolled haemorrhagic shock in swine. Injury. 2012;43:638–47.
37. Chatterjee I. Evolution and biosynthesis of ascorbic acid. Science. 1973;182:1271–2.
38. Cooper CE, Silaghi-Dumitrescu R, Rukengwa M, Alayash AI, Buehler PW. Peroxidase activity of hemoglobin towards ascorbate and urate: a synergistic protective strategy against toxicity of Hemoglobin-Based Oxygen Carriers (HBOC). Biochim Biophys Acta. 2008;1784:1415–20. https://doi.org/10.1016/j.bbapap.2008.03.019.
39. Buehler PW, D'Agnillo F, Hoffman V, Alayash AI. Effects of endogenous ascorbate on oxidation, oxygenation, and toxicokinetics of cell-free modified hemoglobin after exchange transfusion in rat and Guinea pig. J Pharmacol Exp Ther. 2007. https://doi.org/10.1124/jpet.107.126409.
40. Fitzgerald MC, Chan JY, Ross AW, Liew SM, Butt WW, Baguley D, Salem HH, Russ MK, Deasy C, Martin KE, et al. A synthetic haemoglobin-based oxygen carrier and the reversal of cardiac hypoxia secondary to severe anaemia following trauma. Med J Aust. 2011;194:471–3.
41. Baldwin AL, Wiley EB, Summers AG, Alayash AI. Sodium selenite reduces hemoglobin-induced venular leakage in the rat mesentery. Am J Physiol Heart Circ Physiol. 2003. https://doi.org/10.1152/ajpheart.00562.2002.
42. Song BK, Richard Light W, Vandegriff KD, Tucker J, Nugent WH. Systemic and microvascular comparison of lactated Ringer's solution, VIR-HBOC, and alpha-alpha crosslinked haemoglobin-based oxygen carrier in a rat 10% topload model. Artif Cells Nanomed Biotechnol. 2020;48:1079–88.
43. D'Agnillo F, Chang TM. Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties. Nat Biotechnol. 1998;16(7):667–71. https://doi.org/10.1038/nbt0798-667. PMID: 9661202.
44. Simoni J, Simoni G, Wesson DE, Griswold JA, Feola M. A novel hemoglobin-adenosine-glutathione based blood substitute: evaluation of its effects on human blood ex vivo. ASAIO J. 2000;46:679–92. https://doi.org/10.1097/00002480-200011000-00007.
45. Thom CS, Dickson CF, Gell DA, Weiss MJ. Hemoglobin variants: biochemical properties and clinical correlates. Cold Spring Harb Perspect Med. 2013. https://doi.org/10.1101/cshperspect.a011858.
46. Abraham B, Hicks W, Jia Y, Baek JH, Miller JL, Alayash AI. Isolated Hb Providence beta82Asn and beta82Asp fractions are more stable than native HbA(0) under oxidative stress conditions. Biochemistry. 2011;50:9752–66. https://doi.org/10.1021/bi200876e.
47. Strader MB, Bangle R, Parker Siburt CJ, Varnado CL, Soman J, Benitez Cardenas AS, Samuel PP, Singleton EW, Crumbliss AL, Olson JS, Alayash AI. Engineering oxidative stability in human hemoglobin based on the Hb providence (betaK82D) mutation and genetic cross-linking. Biochem J. 2017;474:4171–92. https://doi.org/10.1042/BCJ20170491.
48. Bonaventura J, Bonaventura C, Sullivan B, Ferruzzi G, McCurdy PR, Fox J, et al. Hemoglobin providence. Functional consequences of two alterations of the 2,3-diphosphoglycerate binding site at position beta 82. J Biol Chem. 1976;251:7563–71.
49. Gale RE, Blair NE, Huehns ER, Clegg JB. Hb A-like sickle haemoglobin: Hb S-providence. Br J Haematol. 1988;70:251–2.
50. Alayash AI. Haptoglobin: old protein with new functions. Clin Chim Acta. 2011;412:493–8. https://doi.org/10.1016/j.cca.2010.12.011.
51. Alayash AI, Andersen CB, Moestrup SK, Bülow L. Haptoglobin: the hemoglobin detoxifier in plasma. Trends Biotechnol. 2013;31:2–3. https://doi.org/10.1016/j.tibtech.2012.10.003.
52. Cooper CE, Schaer DJ, Buehler PW, Wilson MT, Reeder BJ, Silkstone G, et al. Haptoglobin binding stabilizes hemoglobin ferryl iron and the globin radical on tyrosine beta145. Antioxid Redox Signal. 2013;18:2264–73. https://doi.org/10.1089/ars.2012.4547.
53. Svistunenko DA, Manole A. Tyrosyl radical in haemoglobin and haptoglobin-haemoglobin complex: how does haptoglobin make haemoglobin less toxic? J Biomed Res. 2019;34:281–91. https://doi.org/10.7555/JBR.33.20180084.

54. Mollan TL, Jia Y, Banerjee S, Wu G, Kreulen RT, Tsai AL, et al. Redox properties of human hemoglobin in complex with fractionated dimeric and polymeric human haptoglobin. Free Radic Biol Med. 2014;69:265–77. https://doi.org/10.1016/j.freeradbiomed.2014.01.030.
55. Jia Y, Wood F, Buehler PW, Alayash AI. Haptoglobin preferentially binds beta but not alpha subunits cross-linked hemoglobin tetramers with minimal effects on ligand and redox reactions. PLoS One. 2013. https://doi.org/10.1371/journal.pone.0059841.
56. Boretti FS, Buehler PW, D'Agnillo F, Kluge K, Glaus T, Butt OI, et al. Sequestration of extracellular hemoglobin within a haptoglobin complex decreases its hypertensive and oxidative effects in dogs and Guinea pigs. J Clin Invest. 2009;119:2271–80. https://doi.org/10.1172/JCI39115.
57. Schaer DJ, Buehler PW, Alayash AI, Belcher JD, Vercellotti GM. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. Blood. 2013;121:1276–84. https://doi.org/10.1182/blood-2012-11-451229.
58. Schaer DJ, Alayash AI. Clearance and control mechanisms of hemoglobin from cradle to grave. Antioxid Redox Signal. 2010;12:181–4. https://doi.org/10.1089/ars.2009.2923.
59. Buehler PW, Karnaukhova E, Gelderman MP, Alayash AI. Blood aging, safety, and transfusion: capturing the "radical" menace. Antioxid Redox Signal. 2011;14:1713–28. https://doi.org/10.1089/ars.2010.3447.
60. Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. Blood. 2014;123:377–90. https://doi.org/10.1182/blood-2013-04-495887.

Nanotechnology-Based Oxygen and Drug Carriers

15

Hans Bäumler, Yu Xiong, and Radostina Georgieva

Background/Introduction

At present, no artificial oxygen transporter is approved in the European Union and the United States. Hemoglobin-based oxygen carriers (HBOC) have been the subject of scientific studies for decades as one of the most promising candidates to succeed. The fabrication of HBOC is sophisticated, but meanwhile also simpler nano- and microparticle fabrication methods exist. One of them consists of three key steps: Co-precipitation of insoluble metal carbonates with biopolymers, Cross-linking of co-precipitated biopolymers and Dissolution of the carbonate templates (CCD-technique). It allows a few-steps fabrication of particles composed of different biopolymers, including hemoglobin, and bioactive agents under mild conditions [1].

A major obstacle to approval has so far been the developers' claim to produce universally applicable blood substitute. However, the restriction to one specific indication seems to be more promising. For example, the approach to transport not only oxygen to the tissue, but also a drug appears to be particularly attractive for the therapy of tumors. The delivery of oxygen to the tumor cells makes them more sensitive in respect of cytotoxic effects of drugs and radiation.

One effect of irradiation of malignant tissue is to generate reactive oxygen species (ROS), which ultimately cause the death of tumor cells [2]. However, the limited oxygen content (hypoxia) in tumor tissue is one of the major drawbacks of cancer treatment by radiation because the presence of oxygen in cancer tissue is fundamental for ROS generation.

H. Bäumler (✉) · Y. Xiong
Institute of Transfusion Medicine, Charité-Universitätsmedizin Berlin, Berlin, Germany
e-mail: hans.baeumler@charite.de; yu.xiong@charite.de

R. Georgieva
Institute of Transfusion Medicine, Charité-Universitätsmedizin Berlin, Berlin, Germany

Department of Medical Physics, Biophysics and Radiology, Medical Faculty, Trakia University, Stara Zagora, Bulgaria
e-mail: radostina.georgieva@charite.de

The mitochondria are the major contributor to the cellular reactive oxygen species (ROS), but oxidative enzymes (e.g., NAPDH oxidases, cyclooxygenases, lipoxygenases or thymidine phosphorylase) also contribute to the cellular ROS pools. ROS are produced by tumor cells but also by cellular components of the tumor microenvironment (TME). The production of ROS by tumor cells plays an important role in driving tumorigenesis. However, ROS production by other infiltrating non-tumor cells, such as tumor-associated fibroblasts as well as the overall oxidative state of the local TME has profound effects on tumor biology.

Reactive oxygen species are important signaling molecules in cancer. While high levels of ROS may cause damage to tissues and cell death, low levels of ROS may have a proliferative effect. In simplified terms, it may be said that a low level of ROS is physiological (transcriptional regulation, differentiation and proliferation), but a medium ROS level leads to enhancement of angiogenesis (tumorigenesis and metastasis). Only high ROS levels result in cytotoxic effects like immune deregulation, apoptosis and autophagy [2].

Therefore, the enhanced oxygen supply to the tumor appears to be a crucial element in radiation therapy. This may be achieved with the help of drug carriers that transport not only cytostatic drugs but also oxygen to the tumor. Hemoglobin based oxygen carriers (HBOC) are the perfect candidate to fulfil these requirements. Additionally to oxygen delivery, their iron content may additionally accelerate the ROS production by converting hydrogen peroxide to the more reactive hydroxyl radical. Simultaneously, they are able carry cytostatic drugs, which are released inside the tumor cell upon lysosomal digestion (Fig. 15.1).

In this chapter, we introduce the CCD-technique as universal approach suitable for the fabrication of biopolymer micro and nanoparticles including HBOC, additionally allowing incorporation of hydrophilic as well as hydrophobic drugs into these particles.

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_15

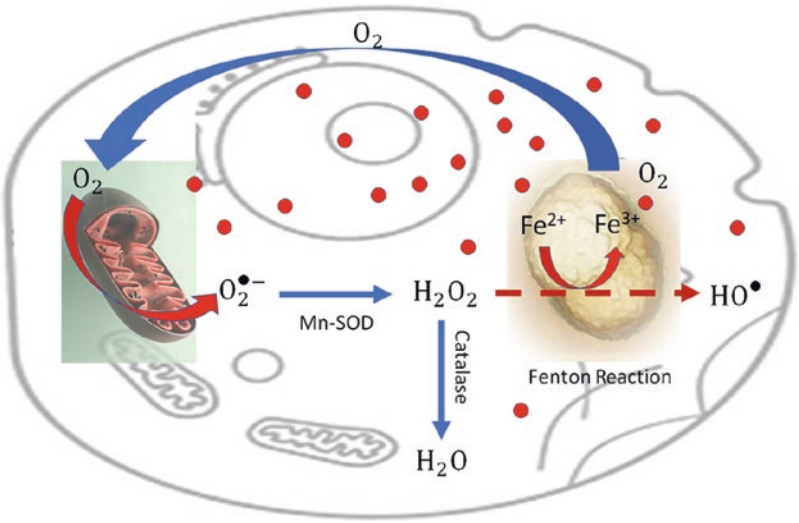


Fig. 15.1 ROS production by mitochondria and HBOC inside tumor cells. HBOC releases oxygen and drugs (red circles symbolize the drug release from HBOC). Mitochondria accept the oxygen and oxygen radicals, which are transformed to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), which may be transferred to water by catalase. But hemoglobin also catalyzes H_2O_2 (Fenton reaction) and produces the very reactive hydroxyl radical

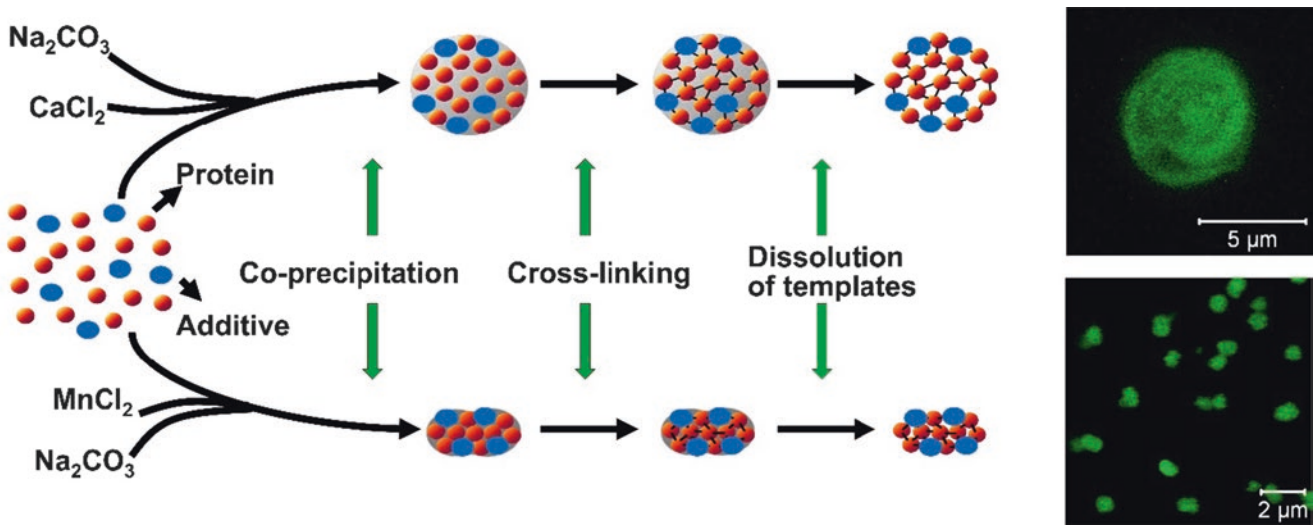


Fig. 15.2 Principle of CCD technique, modified after [5]. The upper line shows the $CaCO_3$ templated formation of hemoglobin microparticle, while the lower line shows the manganese templated submicron particles. The influence of the salt matrix on the particle size is obvious

Nanotechnology Based CCD Technique

The CCD technique was first reported by Bäumler and Georgieva [1]. It allows a few-steps fabrication of particles (Fig. 15.2) composed of different biopolymers and bioactive agents under mild conditions [3–6]. The properties of such particles depend on the fabrication conditions, the choice of the inorganic salts for co-precipitation, the nature of the biopolymers and additives [5]. In the first report of CCD technique, multicompartment particles with concentric compartments loaded independently with different biomolecules, enzymes and magnetic nanoparticles were synthetized [1]. β-glucosidase (β -Glu), glucose oxidase (GOX), and horseradish peroxidase (HRP) have been incorporated in microreactors, where each of these enzymes was located in a separate compartment. The enzyme activity and enzymatic cascade reactions were successfully demonstrated [1].

Oxygen Carriers Fabricated by CCD Technique

Indeed, hemoglobin particles as oxygen carriers and biopolymer particles as drug carriers are successfully synthesized by using this CCD method. $CaCO_3$ templated Hb particles are almost spherical with particle size from 2 to 5 µm and Hb entrapment efficiency from 8% to 50% [3]. The Hb content in the $CaCO_3$ templated Hb particles was roughly one-third of the Hb content of the RBCs [3]. In contrast, $MnCO_3$ templated Hb particles exhibit uniform peanut shape and submicron size with remarkably high Hb entrapment

efficiency of nearly 100% [5]. Addition of human serum albumin (HSA) after co-precipitation reduces Hb entrapment efficiency but prevents particle agglomeration. The Hb content in this HSA protected $MnCO_3$ templated Hb particles was still 80% of the Hb content of the native RBCs [4]. Both types of Hb particles exhibit high oxygen affinity with *p*50 of less than 10 mmHg and low immunogenicity *in vitro* [3, 4]. The deformability of Hb particles was proven by atomic force microscopy (AFM) in the wet and dry state. Hb particles is considered to be non-mutagenic by Ames Test and Mammalian cell gene mutation assay [7]. In the *in vivo* studies, the results of Micronucleous Test in mouse have proven that the supernatant of Hb particles is not mutagenic [7]. Successful perfusions of isolated mouse glomeruli with concentrated Hb particle suspensions were demonstrated *in vitro*. A normal, non-vasoconstrictive behavior of the afferent arterioles is observed, suggesting no oxygen oversupply and limited NO scavenging by these Hb particles fabricated by CCD technique [4].

In a modified procedure, the Hb particle size can be reduced to around 250 nm using sonication [8]. Schakowski et al. prepared Hb based microcapsules coated by albumin by using natural cross-linker genipin instead of toxic glutaraldehyde [9]. To reduce the auto-oxidation of HBOCs, Hu et al. prepared stable Hb polydopamine (Hb-PDA) particles with antioxidative properties based on CCD method and polydopamine modification without glutaraldehyde cross-linking of Hb. Hb-PDA particles were able to exterminate 85% of the hydroxyl radicals and reduce the oxidative injury induced by H_2O_2 [10].

By using the macromolecular cross-linker per-iodate oxidized dextran, the CCD technique was further simplified combining co-precipitation and cross-linking of Hb in one single step since the cross-linker is incorporated into the inorganic template together with Hb [11].

The cross-linking of Hb by glutaraldehyde (GA) generates a certain amount of methemoglobin (MetHb), which is a major obstacle in the production of HBOC. MetHb is a hemoglobin (Hb) derivative with the heme iron in ferric state (Fe^{3+}), unable to deliver oxygen. Quantitative determination of MetHb in HBOC suspensions is indispensable for quality control but common spectrophotometry is not applicable due to the strong light scattering of the particles. Alternatively, ^1H_2O NMR relaxometry was shown to represent a perfect tool for direct measurement of total Hb and MetHb concentrations in samples of hemoglobin submicron particles (Hb-MP) [12]. The longitudinal relaxation rate $1/T_1$ shows a linear increase with increasing MetHb concentration (Fig. 15.3a), whereas the transverse relaxation rate $1/T_2$ linearly increases with the total Hb concentration (Fig. 15.3b). In both linear regressions the determination coefficient (R^2) is higher than 0.99.

The method does not require time-consuming pretreatment or digestion of the particles and is not impaired by light scattering. Therefore, it can be established as the method of choice for the quality control of Hb-MP and similar hemoglobin-based oxygen carriers in the future.

Drug Carriers Fabricated by CCD Technique

The CCD technique is a suitable simple method to form biopolymer drug loaded nano- and microparticles. Several hydrophilic or hydrophobic model drugs were successful incorporated into albumin particles with CCD method. Doxorubicin (DOX) could be effectively loaded into HSA submicron particles fabricated by CCD technique (DOX-HSA-MPs) [13]. The in vitro release of DOX from drug loaded particles in phosphate buffered saline pH 7.4 was less than 1% within 5 h, while up to 40% of the entrapped DOX was released in presence of a protein digesting enzyme mixture (Pronase®) within the same time [13].

Furthermore, using the lung carcinoma cell line A549 as targeting model it was shown that DOX-HSA-MPs were internalized by up to 98% of the cells and were localized in the cell lysosomal compartments (Fig. 15.4). The cellular metabolic activity was significantly reduced after 72 h [13]. Therefore, DOX-HSA-MPs represent a viable alternative for drug delivery application in cancer therapy.

Hydrophobic substances or extracts may also be incorporated in HSA particles by the CCD technique. Jantakee et al. extracted non-sericin (NS) components from *Bombyx mori* silk cocoons. The NS extract contains carotenoids and flavonoids poorly soluble in aqueous media, which were loaded into HSA particles (NS-HSA-MPs) to enhance their bioavailability and biological effects [14].

Riboflavin (RF), a low molecular weight vitamin with low water-solubility was also successfully immobilized in HSA particles (RF-HSA-MPs) (Fig. 15.5) [15].

The RF-HSA-MPs showed good hemocompatibility and the release of RF from the particles exhibits bi-phasic profile with a dominating Fickian diffusion mechanism suggesting that RF-HSA-MPs represent a long-term drug delivery system, and that the CCD-technique of incorporation is applicable to various biomolecules with different molecular weights [15]. By using *ortho*-nitrobenzyl derivative 4-bromomethyl-3-nitrobenzoic acid (BNBA) as a cross-linker, photoresponsive bovine serum albumin (BSA)–BNBA particles may be obtained [16]. This type of particles may be decomposed upon UV irradiation at 365 nm under acidic environment to release the loaded macromolecules. Such a system may be used as potential carriers with stimuli-responsive controlled release of loaded macromolecular drugs [16].

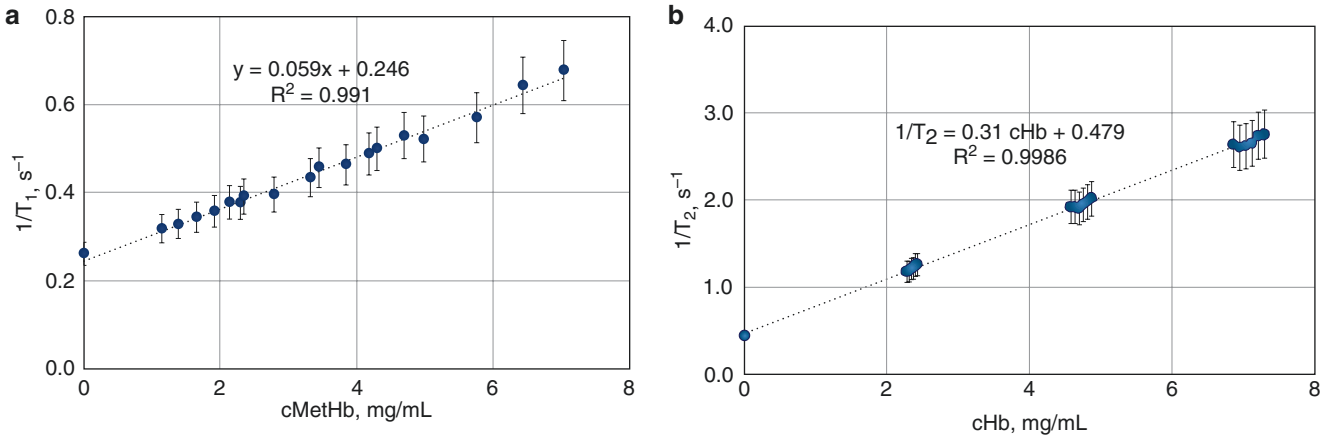


Fig. 15.3 Relaxation rates of Hb-MP/MetHb-MP mixed suspensions prepared with 0.02% GA. Hb-MP and MetHb-MP are mixed at ratios 100/0; 80/20; 60/40; 40/60; 20/80 and 0/100 and diluted to obtain suspensions with PPV of 2, 4 and 6%. (**a**) Longitudinal relaxation rate ($1/T_1$) and (**b**) transverse relaxation rate ($1/T_2$) in dependency on the total Hb concentration in the suspensions. The measurements were performed by using a 0.94T Minispec mq40 relaxometer (Bruker Analytik, Rheinstetten, Germany) operated at a proton frequency of 40 MHz and a preset temperature of 37 °C [12]

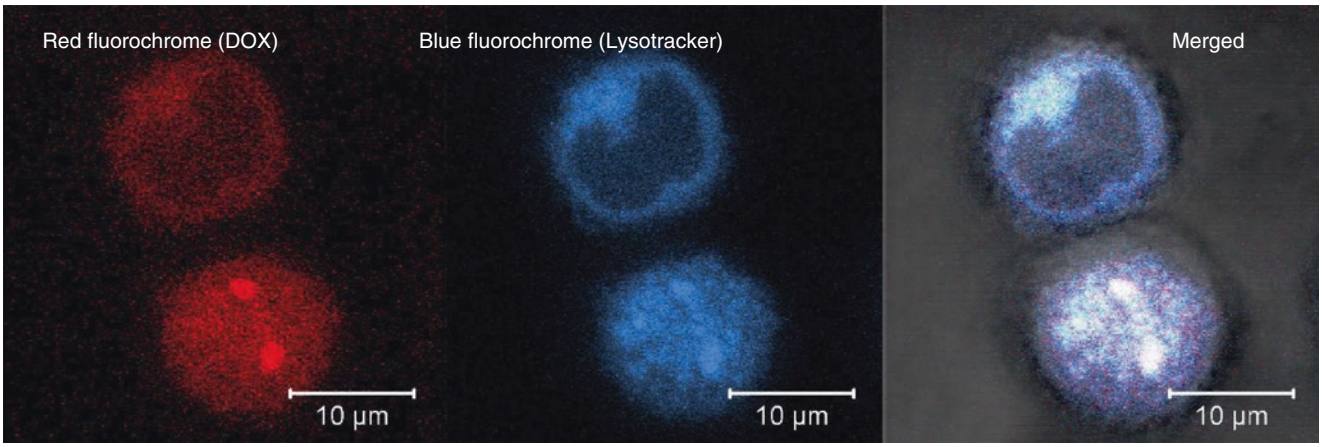


Fig. 15.4 Representative CLSM images in fluorescence and overlay modes of A549 cells after 24 h culturing with DOX-HSA-MPs (5000 particles/cell) and Lysotracker® Deep Red staining [13]

In another modified CCD procedure, GOX and Hb were co-encapsulated with additional multilayer film to create enzymatic cascade microreactors (MRs) [17]. With glucose at physiologically relevant concentrations, the GOX-Hb MRs exhibited a high cascade reaction activity under mild acidic conditions. GOX may catalyze glucose oxidation into gluconic acid and H_2O_2, and the latter may be subsequently decomposed by Hb in the same microreactor to generate hydroxyl radicals (OH·), which significantly inhibited methicillin-resistant Staphylococcus aureus (MRSA) growth and biofilm formation [17].

Summary

Hemoglobin particles fabricated by means of the CCD technique offer many fields of application, because these particles may be used not only for the transport of oxygen but also as drug carrier. Several hydrophilic or hydrophobic drugs like riboflavin or doxorubicin were successfully incorporated into albumin particles. The lung carcinoma cell line A549 was used as targeting model and it was shown that DOX-HSA-MPs were endocytosed and localized in the lysosomal compartment. As a consequence, the cell metabolic activity of the carcinoma cells was significantly reduced.

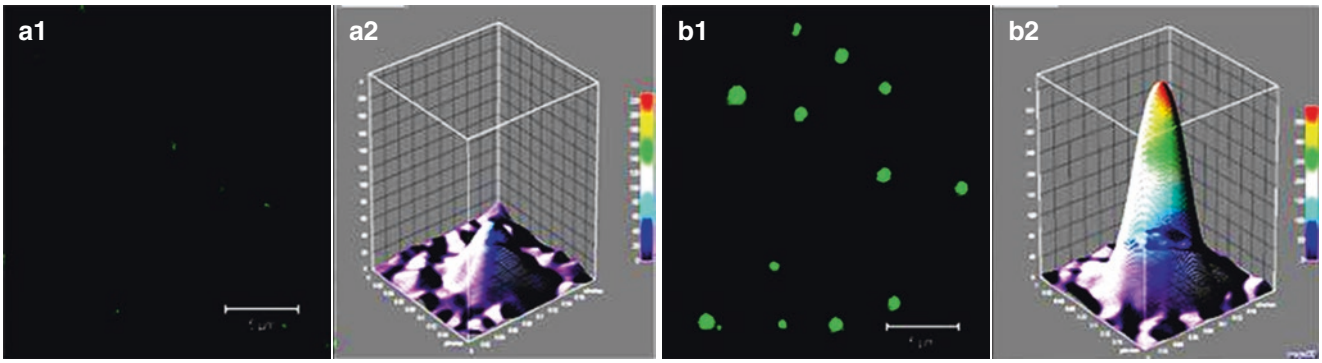


Fig. 15.5 Confocal micrograph of HSA-MPs (**A1**) and RF-HSA-MP (**B1**) in fluorescence mode. Fluorescence emission intensity in 3D color map surface images of (**A2**) HSA-MPs and (**B2**) RF-HSA-MPs [15]

Key Points

Hemoglobin submicron particles carrying oxygen and drugs are suitable for cancer therapy:

- The particles can easily be fabricated by the CCD-technique.
- They are able to enhance ROS generation carrying oxygen and releasing the drug in the tumor cell.
- They are taken up by tumor cells and after digestion of protein particles the drug becomes active.
- The drug is carried directly to the tumor and acts only in its environment in dependence on the surface of particles. Tumor cells with albumin receptors are a favorite target.

References

1. Bäumler H, Georgieva R. Coupled enzyme reactions in multicompartment microparticles. Biomacromolecules. 2010;11:1480–7.
2. Weinberg F, Ramnath N, Nagrath D. Reactive oxygen species in the tumor microenvironment: an overview. Cancers (Basel). 2019;11:1191.
3. Xiong Y, Steffen A, Andreas K, Müller S, Sternberg N, Georgieva R, Bäumler H. Hemoglobin-based oxygen carrier microparticles: synthesis, properties, and in vitro and in vivo investigations. Biomacromolecules. 2012;13:3292–300.
4. Xiong Y, Liu ZZ, Georgieva R, Smuda K, Steffen A, Sendeski M, Voigt A, Patzak A, Bäumler H. Nonvasoconstrictive hemoglobin particles as oxygen carriers. ACS Nano. 2013;7:7454–61.
5. Xiong Y, Georgieva R, Steffen A, Smuda K, Bäumler H. Structure and properties of hybrid biopolymer particles fabricated by co-precipitation cross-linking dissolution procedure. J Colloid Interface Sci. 2018;514:156–64.
6. Bäumler H, Xiong Y, Liu ZZ, Patzak A, Georgieva R. Novel hemoglobin particles-promising new-generation hemoglobin-based oxygen carriers. Artif Organs. 2014;38:708–14.
7. Kao I, Xiong Y, Steffen A, Smuda K, Zhao L, Georgieva R, Pruss A, Bäumler H. Preclinical in vitro safety investigations of submicron sized hemoglobin based oxygen carrier HbMP-700. Artif Organs. 2018;42:549–59.
8. McDonel EM, Hickey R, Palmer AF. Sonication effectively reduces nanoparticle size in hemoglobin-based oxygen carriers (HBOCs) produced through coprecipitation: implications for red blood cell substitutes. ACS Appl Nano Mater. 2020;3:11736–42.
9. Schakowski KM, Linders J, Ferenz KB, Kirsch M. Synthesis and characterisation of aqueous haemoglobin-based microcapsules coated by genipin-cross-linked albumin. J Microencapsul. 2020;37:193–204.
10. Hu J, Wang Q, Wang Y, You G, Li P, Zhao L, Zhou H. Polydopamine-based surface modification of hemoglobin particles for stability enhancement of oxygen carriers. J Colloid Interface Sci. 2020;571:326–36.
11. Kloypan C, Prapan A, Suwannasom N, Chaiwaree S, Kaewprayoon W, Steffen A, Xiong Y, Baisaeng N, Georgieva R, Bäumler H. Improved oxygen storage capacity of haemoglobin submicron particles by one-pot formulation. Artif Cells Nanomed Biotechnol. 2018;46:S964–72.
12. Kaewprayoon W, Suwannasom N, Kloypan C, Steffen A, Xiong Y, Schellenberger E, Pruß A, Georgieva R, Bäumler H. Determination of methemoglobin in hemoglobin submicron particles using NMR relaxometry. Int J Mol Sci. 2020;21:1–13.
13. Chaiwaree S, Prapan A, Suwannasom N, Laporte T, Neumann T, Pruß A, Georgieva R, Bäumler H. Doxorubicin–loaded human serum albumin submicron particles: preparation, characterization and in vitro cellular uptake. Pharmaceutics. 2020;12:1–15.
14. Jantakee K, Prapan A, Chaiwaree S, Suwannasom N, Kaewprayoon W, Georgieva R, Tragoolpua Y, Bäumler H. Fabrication and characterization of human serum albumin particles loaded with non-sericin extract obtained from silk cocoon as a carrier system for hydrophobic substances. Polymers (Basel). 2021;13:334.
15. Suwannasom N, Smuda K, Kloypan C, Kaewprayoon W, Baisaeng N, Prapan A, Chaiwaree S, Georgieva R, Bäumler H. Albumin submicron particles with entrapped riboflavin-fabrication and characterization. Nano. 2019. https://doi.org/10.3390/nano9030482.
16. Li H, Xiong Y, Zhang Y, Tong W, Georgieva R, Bäumler H, Gao C. Photo-decomposable sub-micrometer albumin particles cross-linked by ortho-nitrobenzyl derivatives. Macromol Chem Phys. 2017;218(1700413):1–6.
17. Li T, Li J, Pang Q, Ma L, Tong W, Gao C. Construction of microreactors for cascade reaction and their potential applications as antibacterial agents. ACS Appl Mater Interfaces. 2019;11:6789–95.

Perfluorocarbon-Based Oxygen Carriers

16

Alan D. Kaye, Karla Samaniego, Sumitra Miriyala, Benjamin C. Miller, Elyse M. Cornett, and Steven A. Conrad

Introduction

Transfusion of red blood cells currently remains the most clinically viable means of increasing the oxygen-carrying capacity of blood in the setting of acute or chronic blood loss. Blood transfusion is a common procedure, with over 4.5 million in the US receiving blood transfusions each year, and the number of people in need of blood has surpassed the available donor pool. Not only is there a growing shortage of blood, but blood products are associated with substantial limitations and a low but not insignificant risk [1, 2]. Donated blood is processed to separate the red blood cell component from plasma and platelets. Blood must be stored at a low temperature, has a shelf life of only 42 days, and in most cases must undergo time-consuming compatibility testing to reduce the risk of transfusion reaction, a potentially fatal complication. In cases of acute anemia, an oxygen carrying substitute that can be administered quickly with no risk of incompatibility would have substantial impact in acute and critical care medicine. In chronic anemia the availability of artificial oxygen carriers could reduce the need for perioperative transfusion. Although artificial oxygen carriers would not replace the need for blood, they would help decrease the amount of blood required in many situations that traditionally necessitated blood transfusion.

There are two main categories of artificial oxygen carriers, hemoglobin-based or perfluorocarbon based [3]. These compounds do not provide all the functions of blood such as immune function, coagulation, and acid-base buffering, and therefore are not true blood substitutes. Instead, they replicate only the oxygen-carrying function of erythrocytes, and the term artificial oxygen carriers is preferred. This chapter will focus on the use of perfluorocarbons as oxygen carriers for support of oxygen transport to tissue and related applications.

Chemical and Physical Properties

Perfluorocarbons refer to a family of compounds that are hydrocarbons with fluorine atoms substituted for all hydrogen atoms, with a chemical structure abbreviated C_xF_y. Fluorine is the most electronegative element with one of the highest electron affinities and low polarizability. The chemical properties of fluorine contribute to the multiple physiological attributes that make perfluorocarbon-based oxygen carriers appealing to use as artificial oxygen carriers.

The low polarizability of fluorine and its slightly larger size compared to hydrogen makes perfluorocarbons conformationally different from their hydrocarbon counterparts. In contrast

A. D. Kaye (✉)
Department of Anesthesiology and Department of Pharmacology, Toxicology, and Neurosciences, LSU School of Medicine, Shreveport, LA, USA

Department of Anesthesiology and Pharmacology, LSU School of Medicine, New Orleans, LA, USA

Department of Anesthesiology and Pharmacology, Tulane School of Medicine, New Orleans, LA, USA
e-mail: alan.kaye@lsuhs.edu

K. Samaniego
Department of Surgery, Texas Tech University Health Sciences Center, School of Medicine, Lubbock, TX, USA
e-mail: karla.samaniego@ttuhsc.edu

S. Miriyala
Department of Cellular Biology and Anatomy, Louisiana State University Health Shreveport, Shreveport, LA, USA
e-mail: sumitra.miriyala@lsuhs.edu

B. C. Miller
Louisiana State University Health Shreveport, Shreveport, LA, USA
e-mail: bmil10@lsuhsc.edu

E. M. Cornett
Department of Anesthesiology, LSU Health Shreveport, Shreveport, LA, USA
e-mail: elyse.bradley@lsuhs.edu

S. A. Conrad
Departments of Medicine, Emergency Medicine, Pediatrics and Surgery, Louisiana State University Health Shreveport, Shreveport, LA, USA
e-mail: steven.conrad@lsuhs.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_16

to hydrocarbons that have linear, zig-zag shapes and demonstrate conformational flexibility, perfluorocarbons based on this backbone take on a helical shape related to the steric repulsion of fluorine and become rigid, rod-like structures [4, 5]. The electronegativity of fluorine results in a strong C—F bond that is enhanced by the presence of adjacent bonds, yielding chemical and thermal stability, further strengthened by a dense electron sheath. The C—F bond is extremely polar; however, this does not result in polarization of the overall molecule as one would expect. In fact, perfluorocarbons are among the most nonpolar solvents that exist because all the dipole moments within the same molecule cancel each other out, which makes the overall compound nonpolar [4, 5].

The low polarizability of fluorine accounts for the weak intermolecular forces witnessed in perfluorocarbons. Because perfluorocarbons are not as densely packed as their hydrocarbon counterparts, they have a greater ratio of surface area to volume occupied in water; this results in weak van der Waals interactions with water and thus highly hydrophobic nature of perfluorocarbons [4, 6]. Furthermore, the high polarity of C—F bonds prevents the formation of the induced dipoles and van der Waals forces necessary for lipid solubility, meaning perfluorocarbons are highly lipophobic as well [4, 7]. As a result of their simultaneous hydrophobic and lipophobic qualities, perfluorocarbons are biologically inert. They are not metabolized by the body and are removed by the reticuloendothelial system and via the lungs during exhalation [4, 8]. An example of their low water solubility and biological inertness in practice is how perfluorocarbons are used in commercially available contrast agents. These traits allow for the stabilization of injectable microparticles and prolong the existence of these contrast agents in vivo for ultrasonography [4, 9].

As noted, intermolecular forces between perfluorocarbon molecules are weak, yielding viscosities lower than water but higher densities, as much as twice that of water, and low surface tension. Due to their hydrophobic properties they are not miscible with water or blood [10]. In general perfluorocarbons are biologically and chemically inert but certain compounds can exhibit toxicity *in vivo*. Some perfluorocarbons have other halogens such as bromine partially substituted that alters some of the properties such as radiolucency.

Perfluorocarbons have high solubility for gases, including oxygen and carbon dioxide due to their weak intermolecular interactions, thereby their interest for use as artificial oxygen carriers [11]. In contrast to hemoglobin, perfluorocarbons are chemically inert and do not chemically bind to respiratory gases. The strong C—F bond results in stabilization of the perfluorocarbon molecule, and the steric shielding of the central carbon atom by fluorine atoms adds additional kinetic stability and inhibit reactivity [4, 12]. Because they are inert to oxidation, perfluorocarbons remain functional in the presence of carbon monoxide (CO) and even accelerate the recovery from CO poisoning by transporting excess CO [7, 13, 14]. Being chemically inert to respiratory gases also makes perfluorocarbons useful in the treatment of decompression sickness, which is caused by the supersaturation of respiratory gases, primarily nitrogen in blood and tissues followed by rapid decompression. Nitrogen comes out of solution as bubbles that obstruct small vessels. Animal studies have shown that perfluorocarbons have been effective for washing out nitrogen and preventing embolism [7, 15].

Perfluorocarbon Compounds

Perfluorocarbons may be classified into five categories based on the primary perfluorocarbon backbone utilized in the product: (1) perfluorodecalin, (2) perfluorooctyll bromide, (3) tertbutylperfluorocyclohexane, (4) dodecafluoropentane and (5) perftoran [7]. A summary of characteristics of these perfluorocarbons is provided in Table 16.1. These perfluorocarbons have been manufactured in several compounds for clinical development.

Table 16.1 Perfluorocarbon compounds for oxygen carriage and related uses

Product class	Perfluorodecalin	Perfluorooctyll bromide	Perfluoro tert-butylcyclohexane	Dodecafluoropentane
Formula	$C_{10}F_{18}$	C_8BrF_{17}	$C_{10}F_{20}$	C_5F_{12}
Configuration	Cyclic	Linear	Cyclic	Linear
Oxygen solubility	403 mL/L	527 mL/L		800 mL/L
Representative compounds	Fluosol-DA (20% emulsion) Perftoran	Perflubron Oxygent Imagent	Oxycyte	APF-30M
Clinical investigations	Artificial oxygen carrier in resuscitation Percutaneous coronary angioplasty (PCA) Occlusive vascular disease	Injectable contrast agent Liquid ventilation Pulmonary lavage	Traumatic brain injury	Acute ischemic stroke
US Regulatory status	Approval 1989 for PCA, discontinued 1994	Approval as contrast agent 1993, now discontinued	Not approved	Not approved

Fluosol-DA was granted FDA approval for coronary angioplasty in 1989, but the approval was later withdrawn in 1994 due to a limited shelf life and side effects related to complement activation [7]. Perftoran, a 7:3 mixture of perfluorodecalin and perfluoro-N-(4-methylcyclohexyl)-piperidine, was approved in several countries from 2005 to 2010 to treat blood loss, but isn't currently approved for use [7]. Oxygent and Oxycyte reached human trials in the USA but, as of now, haven't been approved by the FDA for use [7]. Oxycyte successfully completed phase II trials but was abandoned by the sponsor in 2014 due to lack of patient enrollment and financial reasons. Oxygent reached phase III trials, was transiently abandoned because of safety issues that later were disproved to be product-related [7]. During the initial phase III trial, it was thought that Oxygent caused excess bleeding and neurological events such as stroke-causing Alliance to suspend the trial [11]. Subsequent analysis of the clinical study data concluded that the conduct of the study and not the emulsion was responsible for the observed adverse events. It was demonstrated that the neurological and bleeding events were caused by excessive hemodilution in the patients receiving Oxygent instead of by Oxygent itself [11]. Furthermore, it was shown that inadequate management of blood pressure during the rapid autologous blood harvesting procedure resulted in decreased perfusion to the brain in some of these patients as well [11]. Related to these findings, new trials are being initiated in China and parts of Europe [7].

Perfluorocarbon-Based Oxygen Delivery

In 1966 Clark and Gollan were the first to demonstrate that perfluorocarbon liquids have a tremendous capacity for carrying and delivering oxygen [4, 7, 11]. Perfluorodecalin, one of the most studied perfluorocarbons, has 1000 times the molecular solubility for oxygen than that of water [7]. The combination of strong intramolecular forces with weak intermolecular forces allows perfluorocarbon liquids to behave almost like ideal fluids in that they're easily able to dissolve gases with similar, low cohesivity properties such as O2, CO2, NO, etc. [4]. The mechanism of perfluorocarbons using loose van der Waals forces to dissolve oxygen for delivery contrasts with the way hemoglobin transports oxygen, using chemical bonding between the oxygen molecule and the iron atom in heme. Oxygen uptake by perfluorocarbon solutions is linear in contrast to the sigmoidal uptake by hemoglobin characteristic of the varying affinity for oxygen as oxygen is bound [11]. This linear relationship represents perfluorocarbon solubility following Henry's law, which states that the amount of gas dissolved in a liquid is directly proportional to the partial pressure of the gas. One consequence of following Henry's law is that having an elevated arterial oxygen partial pressure maximizes the benefits of the oxygen-carrying capacity of perfluorocarbon solutions [4]. Another consequence is the rapid oxygen uptake by perfluorocarbons and the rapid and extensive oxygen extraction when needed by tissues [4, 5, 7]. When compared to RBCs, oxygen loading and unloading by perfluorocarbons occurs at twice the rate, and the rate of oxygen extraction from perfluorocarbons is three times higher because perfluorocarbons can release over 90% of loaded oxygen [4, 16]. Furthermore, when perfluorocarbon emulsions are added to the plasma, the solubility of oxygen is increases by factors of 10–50 [17, 18].

Related to their hydrophobic and immiscible nature, perfluorocarbons cannot be directly administered intravenously but must be emulsified to generate thermodynamically metastable but kinetically stable mixtures [9]. Characteristics desirable for intravascular oxygen transport include emulsion stability and low vapor pressure with rapid excretion. These ideal properties are not common among perfluorocarbons, but those with slightly lipophilic properties such as F-octyl bromide (perflubron) are more suitable.

While perfluorocarbons have several attractive features for potential use as RBC substitutes in the setting of blood loss and surgery, the hydrophobic nature of perfluorocarbons that make them immiscible in aqueous media such as blood. To use perfluorocarbons in the setting of acute blood loss, perfluorocarbons must be manufactured into a stable, emulsified, or encapsulated form for intravascular use [4, 5, 16]. This has led to the development of multiple generations of perfluorocarbon formulations.

Clinical Applications

Research into RBC substitutes has become increasingly important given the limited availability of RBC concentrates and the growing demand to accommodate an aging population [19, 20]. Utilizing purely synthetic compounds as oxygen carriers has the goal to provide a substitute with an unlimited supply, universal compatibility, lack of disease transmittance, and avoidance of transfusion-related reactions and complications [7, 16].

One of the first-generation perfluorocarbons emulsions developed was Fluosol-DA, which is a perfluorodecalin product incorporating Pluronic-68 and egg yolk phospholipids for emulsification. Fluosol-DA is the only perfluorocarbon to have achieved FDA approval, available from 1989 to 1994, with an indication for percutaneous transluminal coronary balloon angioplasty for reperfusion of ischemic tissue [21, 22]. First-generation perfluorocarbon emulsions had several drawbacks such as less effectiveness in oxygen delivery, short intravascular half-life, storage at freezing temperatures, and complement activation by emulsifying agents [22]. Second-generation perfluorocarbon emulsions like

Oxygent, a perfluorooctyl bromide-based product, addressed some of these issues by having a higher perfluorocarbon content, using natural phospholipids for emulsification, and not requiring freezing for storage [22]. However, these products had similar side effects such as the cytokine-mediated processes and platelet sequestration [22].

One promising product to treat hemorrhagic shock that has milder adverse effects is Perftoran. Created in Russia and then approved for use in 1996 to treat hemorrhage and various ischemic conditions, Perftoran is a perfluorodecalin-based product that uses Proxanol 268 instead of egg yolk phospholipid as the emulsifier [23]. According to reports, Perftoran has been administered over 35,000 times and has demonstrated increased benefit with milder adverse events reported due to the product's smaller size [23]. As of now, FluorO2 Therapeutics, Inc plans to manufacture Perftoran (rebranded as Vidaphor) in the United States for clinical trials [24]. Another promising perfluorocarbon solution being investigated is composed of a perfluorodecalin core surrounded by a biocompatible albumin capsule. These albumin-derived perfluorocarbon solutions demonstrated functionality as artificial oxygen carriers and biocompatibility by lacking severe side effects in animal models [11]. An additional pre-clinical study demonstrated that these perfluorocarbon solutions acted as life-saving RBC substitutes in the setting of massive hemodilution [25]. Although there are currently no perfluorocarbon solutions being regularly used for humans as the setting of blood substitution, clinical investigations are still underway. Further improvements in perfluorocarbon products may allow for synthetic blood substitutes in the future.

The use of perfluorocarbon-based oxygen carriers, used in the setting of other blood-saving techniques would allow for surgical procedures with risk of blood loss to be performed while simultaneously avoiding or reducing the amount of allogeneic transfusion [3]. A major field of application for perfluorocarbons is clearly the replacement of blood transfusions in trauma or surgical setting requiring large volume blood loss [5].

Future Directions

Liquid Ventilation

Humans, along with other air-breathing species, exchange oxygen and carbon dioxide through lungs, a respiratory organ with an air-blood interface. In fish and other vertebrates and invertebrates, gills serve as the respiratory organ able to extract oxygen from water. In human acute lung injury, the resulting inflammatory injury to the alveolar epithelium and loss of surfactant with elevation of alveolar surface tension leads to alveolar collapse and/or filling of alveoli with edema fluid. It was hypothesized that filling the lungs with fluid rather than air would eliminate the air-fluid interface (and surface tension along with it), reduce atelectasis, allow restoration of alveolar structure, and improve gas exchange. For this to be successful, the fluid used for installation into the lungs would have to be capable of carrying sufficient oxygen to meet oxygen transfer needs. Perfluorocarbons have the characteristics required to meet these needs. The use of perfluorocarbons in this manner is termed liquid ventilation, wherein the lungs are insufflated with an oxygenated perfluorochemical liquid rather than an oxygen-containing gas mixture. The use of perfluorocarbons as the biochemically inert carrier of oxygen and carbon dioxide, rather than nitrogen, has several theoretical benefits for treating acute lung damage.

There have been two major approaches to liquid ventilation: total liquid ventilation and partial liquid ventilation. *Total liquid ventilation* involves filling all potential air spaces including airway dead space volume with liquid. Perfluorocarbons have a kinematic viscosity close to that of water and are roughly twice as dense as water. Since perfluorocarbons are denser and more viscous than gas, with a slower spreading rate and higher diffusion coefficients, the work of liquid breathing exceeds the respiratory muscles to maintain ventilation for extended periods of time. A special mechanical ventilator to deliver liquid tidal volumes, replenish oxygen in the perfluorocarbon, and remove carbon dioxide are necessary to maintain pulmonary gas exchange [26]. Total liquid ventilation is a technologically challenging process that is still in pre-clinical experimentation and evaluation and has not entered clinical trials.

Partial liquid ventilation differs from total liquid ventilation by filling only the functional residual capacity with perfluorocarbon (approximately 30 mL/kg) and using a conventional mechanical ventilator to deliver gas tidal volumes. This approach entered early clinical trials using perflubron as the perfluorocarbon [27] but was associated with complications such as barotrauma related to the large tidal volumes that were common at the time.

Non-respiratory applications such as pulmonary medication administration and radiographic imaging may have potential clinical use [20].

Albumin-Derived Perfluorocarbon Solutions

Most hemoglobin-based artificial oxygen carriers were associated with severe side effects in clinical trials and therefore further development was suspended. While hemoglobin-based oxygen carriers must maintain a specific size to avoid local nitric oxide scavenging from the endothelium, perfluorocarbon-based oxygen carrier emulsified particles tend to grow because of Oswald ripening and flocculation. Wrobeln

et al., successfully synthesized nanoscaled perfluorocarbon-based oxygen carriers with a perfluorodecalin core surrounded by a biocompatible albumin shell (capsules) [14]. In a rat model of large-scale hemodilution with exchange of approximately 95% of the blood volume with either capsules in a plasma-like solution (treatment) or the plasma-like solution without capsules (control), hypoxia sensitive organs such as the small intestine and kidney were protected. This study served as a "proof of concept" that capsules are a potentially life-saving erythrocyte substitute. With these capsules tissue hypoxia, even at critically low hematocrit, can be avoided [25].

Summary

Artificial oxygen carriers are divided into two categories: one based on hemoglobin and the second based on perfluorocarbons. Neither are a complete substitute for blood but do provide the ability to carry clinically useful amounts of oxygen to tissues. Perfluorocarbons have been investigated as oxygen carriers in a range of biological applications for several decades. These compounds are chemically and physiologically inert, have temperature and storage stability, provide little to no infectious risk, and have well-established gas transport properties. Perfluorocarbons are gas-dispersing chemicals that are not suited for direct injection into the vascular system but require preparation such as emulsification or other stable nanoparticle generation. When used in conjunction with other blood-saving measures, perfluorocarbon-based oxygen carriers have the potential to compensate for blood loss during surgical operations while avoiding or reducing the need for allogeneic transfusion.

References

1. Delaney M, Wendel S, Bercovitz RS, Cid J, Cohn C, Dunbar NM, et al. Transfusion reactions: prevention, diagnosis, and treatment. Lancet. 2016;388(10061):2825–36.
2. Goodnough LT. Risks of blood transfusion. Crit Care Med. 2003;31(12 Suppl):S678–86.
3. Henkel-Hanke T, Oleck M. Artificial oxygen carriers: a current review. AANA J. 2007;75(3):205–11.
4. Riess JG. Understanding the fundamentals of perfluorocarbons and perfluorocarbon emulsions relevant to in vivo oxygen delivery. Artif Cells Blood Substit Immobil Biotechnol. 2005;33(1):47–63.
5. Spahn DR. Blood substitutes. Artificial oxygen carriers: perfluorocarbon emulsions. Crit Care. 1999;3(5):R93–7.
6. Dalvi VH, Rossky PJ. Molecular origins of fluorocarbon hydrophobicity. Proc Natl Acad Sci U S A. 2010;107(31):13603–7.
7. Jagers J, Wrobeln A, Ferenz KB. Perfluorocarbon-based oxygen carriers: from physics to physiology. Pflugers Arch. 2021;473(2):139–50.
8. Haldar R, Gupta D, Chitranshi S, Singh MK, Sachan S. Artificial blood: a futuristic dimension of modern day transfusion sciences. Cardiovasc Hematol Agents Med Chem. 2019;17(1):11–6.
9. Schutt EG, Klein DH, Mattrey RM, Riess JG. Injectable microbubbles as contrast agents for diagnostic ultrasound imaging: the key role of perfluorochemicals. Angew Chem Int Ed Engl. 2003;42(28):3218–35.
10. Lemal DM. Perspective on fluorocarbon chemistry. J Org Chem. 2004;69(1):1–11.
11. Riess JG. Perfluorocarbon-based oxygen delivery. Artif Cells Blood Substit Immobil Biotechnol. 2006;34(6):567–80.
12. Kirsch P. Modern fluoroorganic chemistry: synthesis, reactivity, applications. 2nd ed. John Wiley & Sons, Inc; 2013.
13. Yokoyama K. Effect of perfluorochemical (PFC) emulsion on acute carbon monoxide poisoning in rats. Jpn J Surg. 1978;8(4):342–52.
14. Wrobeln A, Laudien J, Gross-Heitfeld C, Linders J, Mayer C, Wilde B, et al. Albumin-derived perfluorocarbon-based artificial oxygen carriers: a physico-chemical characterization and first in vivo evaluation of biocompatibility. Eur J Pharm Biopharm. 2017;115:52–64.
15. Mayer D, Ferenz KB. Perfluorocarbons for the treatment of decompression illness: how to bridge the gap between theory and practice. Eur J Appl Physiol. 2019;119(11–12):2421–33.
16. Khan F, Singh K, Friedman MT. Artificial blood: the history and current perspectives of blood substitutes. Discoveries (Craiova). 2020;8(1):e104.
17. Ferenz KB, Steinbicker AU. Artificial oxygen carriers-past, present, and future-a review of the most innovative and clinically relevant concepts. J Pharmacol Exp Ther. 2019;369(2):300–10.
18. Spiess BD. Perfluorocarbon gas transport: an overview of medical history with yet unrealized potentials. Shock. 2019;52(1S Suppl 1):7–12.
19. Eggleton CD, Roy TK, Popel AS. Predictions of capillary oxygen transport in the presence of fluorocarbon additives. Am J Physiol. 1998;275(6):H2250–7.
20. Wrobeln A, Schluter KD, Linders J, Zahres M, Mayer C, Kirsch M, et al. Functionality of albumin-derived perfluorocarbon-based artificial oxygen carriers in the Langendorff-heart (dagger). Artif Cells Nanomed Biotechnol. 2017;45(4):723–30.
21. Bachert SE, Dogra P, Boral LI. Alternatives to transfusion. Am J Clin Pathol. 2020;153(3):287–93.
22. Williamson LM, Devine DV. Challenges in the management of the blood supply. Lancet. 2013;381(9880):1866–75.
23. Castro CI, Briceno JC. Perfluorocarbon-based oxygen carriers: review of products and trials. Artif Organs. 2010;34(8):622–34.
24. Latson GW. Perftoran (Vidaphor)-Introduction to Western medicine. Shock. 2019;52(1S Suppl 1):65–9.
25. Jahr JS, Guinn NR, Lowery DR, Shore-Lesserson L, Shander A. Blood substitutes and oxygen therapeutics: a review. Anesth Analg. 2021;132(1):119–29.
26. Wrobeln A, Jagers J, Quinting T, Schreiber T, Kirsch M, Fandrey J, et al. Albumin-derived perfluorocarbon-based artificial oxygen carriers can avoid hypoxic tissue damage in massive hemodilution. Sci Rep. 2020;10(1):11950.
27. Hirschl RB, Conrad S, Kaiser R, Zwischenberger JB, Bartlett RH, Booth F, et al. Partial liquid ventilation in adult patients with ARDS: a multicenter phase I-II trial. Ann Surg. 1998;228(5):692–700.

Platelet Substitutes

17

Chancellor Donald and Marc J. Kahn

Introduction

Platelets are the cellular blood component that facilitates hemostasis through the processes of platelet adhesion, activation and aggregation. Every year in the United States, over 2 million units of platelet components are transfused [1]. Circulating platelets are derived from the membrane of megakaryocytes in the bone marrow and as such do not have a nucleus. Platelets are small discoids, measuring 2–3 mcm in diameter, and upon activation, develop projections of their outer membranes which assist in forming a stable clot. Platelets contain alpha and dense granules that aid in the formation of a clot when they release their procoagulant contents. Alpha granules contain clotting regulators including factors V, and VIII as well as fibrinogen, while dense granules contain platelet activators such as ADP, calcium and serotonin.

Adhesion of platelets to damaged endothelium occurs when collagen is exposed. Platelets tether to damaged endothelium using the platelet receptor GP Ib-IX-V and the ligand, von Willebrand factor (vWF). Exposure of collagen allows for platelet binding via GP VI and the adhesion molecule α2β1. Adhered platelets are the basis of primary hemostasis. Following adhesion to collagen, platelets become activated. Activation involves a complex series of mechanisms including "inside-out" signaling where the platelet membrane and receptors become primed for aggregation, and "outside-in" signaling where the microenvironment facilitates changes in platelet membrane receptors to facilitate formation of a clot. Platelet activation is also characterized by platelet production of thromboxane A2, tissue factor binding to factor VII, generation of thrombin, and platelet release of ADP. Platelet activation is followed quickly by platelet aggregation which activates the platelet fibrinogen receptor GP α2b-β3. Fibrinogen and its receptor act to form a multilayered platelet aggregate to plug a damaged blood vessel. Additionally, activation promotes the release of platelet granule contents that act to further recruit more platelets to the site of vascular injury. Finally, the platelet phospholipid surface acts as the substrate for several important steps in the clotting cascade. The result is the formation of a stable fibrin clot.

In addition to their role in hemostasis, it has become increasingly recognized that platelets also contribute to both inflammation and the immune response. These functions will not be discussed further in this chapter that will instead focus on platelet substitutes to aid in hemostasis and thrombosis.

Given the need for hemostatic control in large cohorts of patients, the development of platelet substitutes continues to be an area of active research. The topic of platelet substitutes can be divided into the creation of modified erythrocyte derivatives, the use of non-cellular nanoparticles to promote hemostasis and thrombosis, the use of non-cellular hemostatic agents to improve platelet function, and the use of thrombopoietic agents to increase platelet numbers in thrombocytopenic patients.

Modified Erythrocytes

The major determinant of platelet aggregation is the ability of fibrinogen to bind platelets together using the platelet integrin receptor GPα2b-β3. There are over 80,000 of these receptors on each platelet capable of binding over 40,000 fibrinogen molecules [2]. The receptor, GPα2b-β3, recognizes the amino acid sequence, arginine-glycine-aspartic acid (RGD) found in fibrinogen. In the early 1990s, Coller, et al., created thromboerythrocytes, one of the earliest examples of a synthetic platelet. Thromboerythrocytes were red

C. Donald
Tulane University School of Medicine, Department of Medicine, Division of Hematology/Medical Oncology, New Orleans, LA, USA

M. J. Kahn (✉)
Kirk Kerkorian School of Medicine, University of Nevada Las Vegas, Office of the Dean, Las Vegas, NV, USA
e-mail: marc.kahn@unlv.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_17

cells modified to include peptides with the RGD sequence [3]. These constructs contained a seven amino acid peptide covalently linked to erythrocytes at a high enough concentration to facilitate aggregation. Thromboerythrocytes were found to aggregate with normal platelets and to aggregate with GPα2b-β3 found on normal platelets. Fortunately, in an animal model there was minimal hemolysis when thromboerythrocytes were introduced into the circulation. Although novel, unfortunately, thromboerythrocytes were found to be immunogenic, limiting their clinical utility [4].

In addition to red cell membranes, liposomes are an attractive substrate to use to create bioengineered platelet substitutes as they are easily introduced and maintained in the circulation and can incorporate hydrophobic as well as hydrophilic moieties. Plateletsomes, liposomes containing various platelet membrane proteins including GPα2b-β3, GP Ib, and GPVI/III have been developed [5]. Unfortunately, to date, these products have been rapidly cleared from the circulation by the spleen, greatly limiting their therapeutic efficacy [6]. Additionally, some liposomal preparations were found to lose stability while being stored over time [7].

Non-Cellular Hemostatic Agents

As previously described, fibrinogen is an essential protein in thrombus formation and platelet aggregation. The RGD (Arg-Gly-Asp) sequence within fibrinogen binds to a platelet integrin on GP α2b-β3 and is employed in the creation of modified erythrocytes as described above. Another class of sequences in the fibrinogen protein that bind to GP α2b-β3 is ^{400}HHLGGAKQAGDV^{411}, called H12, the fibrinogen γ-chain dodecapeptide, in the carboxyl-terminal of the c-chain [8]. H12 was conjugated to the surface of polymerized albumin particles modified with polyethylene glycol chains to produce blood-compatible particles (H12–PEG–polyAlb) that had prolonged blood residence time and enhanced stability *in vitro* and *in vivo*. The H12–PEG–polyAlb significantly shortened the ear bleeding time of severely thrombocytopenic rabbits and showed no effect on the inhibition or promotion of endogenous and exogenous coagulation activities [9].

Inert beads or formaldehyde-fixed platelets bearing fibrinogen on their surfaces have been observed to enhance platelet aggregation [10]. This has resulted in the development of two kinds of fibrinogen coated albumin particles (FAM).

Thrombospheres are a FAM preparation with a poorly understood mechanism of action, but with a demonstration of shortened microvascular ear bleeding time in thrombocytopenic rabbits and a duration of action of at least 72 hours after injection. In normal rabbits, thrombospheres were not found to be thrombogenic and did not shorten platelet survival [11].

Synthocytes represent the other FAM preparation. Regarding the mechanism of action, experiments with normal and thrombocytopenic human blood in an endothelial cell matrix-coated perfusion chamber demonstrated an interaction between the fibrinogen-coated albumin microcapsules and native platelets. It was shown that the fibrinogen-coated albumin microcapsules could facilitate platelet adhesion to endothelial cell matrix and correct the impaired formation of platelet aggregates in relatively platelet-poor blood. No potential systemic prothrombotic effect of the microcapsules was observed in a model of rabbit venous thrombosis [12].

A novel platelet substitute consisting of disk-shaped nanosheets having a large contact area for the targeting site, rather than conventional small contact area spherical carriers has also been developed. The H12 peptide was conjugated to the surface of the free-standing nanosheets made of biodegradable poly(d,l-lactide-co-glycolide) (PLGA). The resulting H12-PLGA nanosheets specifically interacted with the activated platelets adhered on a collagen surface at twice the rate of the H12-PLGA microparticles under flow conditions, and showed platelet thrombus formation in a two-dimensional spreading manner [13]. To date, none of the non-cellular platelet substitutes have been sufficiently studied in patients to warrant FDA approval.

Agents to Improve Platelet Function

The antifibrinolytic lysine analogues tranexamic acid and aminocaproic acid have been explored as adjuncts to prophylactic platelet transfusions. These medications help to stabilize the clots that form after bleeding, therefore reducing the chances of further bleeding as well as possibly the need for transfusing platelets. A meta-analysis indicated that the evidence available for the use of antifibrinolytics in hematology patients at risk for bleeding is very limited. The trials were too small to assess whether or not antifibrinolytics decrease bleeding or increased the risk of thromboembolic events [14].

The effects of prophylactic tranexamic acid as an adjunct to routine transfusion therapy on bleeding and transfusion requirements was evaluated in a multicenter, double blind placebo controlled randomized clinical trial in patients undergoing treatment for hematologic malignancy anticipated to have hypoproliferative thrombocytopenia. The study revealed that prophylactic tranexamic acid had no effect on the incidence of WHO Grade 2+ bleeding when given in addition to routine platelet transfusions to severely thrombocytopenic patients undergoing therapy for hematologic malignancy. An increased incidence of line occlusion in the tranexamic acid arm was observed but no increase in other types of thrombotic events was detected [15].

Anecdotally, recombinant activated factor VII (rFVIIa) has been reported to control bleeding in patients with thrombocytopenia [16]. Before it can be considered appropriate for use, properly designed clinical trials will be required to fully evaluate the efficacy of rFVIIa in the treatment of thrombocytopenic patients.

Thrombopoietic Agents

As noted, despite intense effort, the creation of a true hemostatic platelet substitute that could survive in the circulation and provide hemostasis has remained elusive. For thrombocytopenic patients, interventions designed to increase the production of natural platelets in the bone marrow have proven a better solution.

Interleukin 11 (IL-11) is a cytokine the promotes megakaryocyte maturation. Discovered in 1990, it was marketed as the drug oprelvekin to improve platelet recovery after chemotherapy. Oprelvekin was the first agent in the class of thrombopoietics [17].

Following the identification and cloning of thrombopoietin (TPO) as the agent that supports megakaryocyte proliferation and differentiation into platelet-producing cells, several TPO analogues have been developed for clinical use [18]. Early studies of thrombopoietin receptor agonists including full-length TPO, were unsuccessful due to antibody formation that exhibited cross reactivity with endogenous TPO [19]. Second generation agents included the small molecule eltrombopag and the peptibody, romiplostim. Both of these are approved to increase platelet counts following chemotherapy and in patients with immune thrombocytopenic purpura (ITP) or hepatitis C. A third-generation molecule, avatrombopag, has been approved in patients with ITP and in the treatment of periprocedural thrombocytopenia in patients with chronic liver disease [20]. Lusutrombopag is another approved oral small molecule to increase platelet counts. There are other agents under investigation with similar mechanisms of action. These drugs are safe, with their major side effect being complications from thrombocytosis. In the absent of a true synthetic platelet, these agents are important in inducing innate platelet production in patients with thrombocytopenia.

Conclusion

There is currently no available true synthetic platelet. Perhaps, as nanotechnology techniques becomes more advanced, a true platelet substitute can be developed. Until then, for thrombocytopenic patients, it is best to develop molecules that facilitate megakaryocyte proliferation and platelet production in the bone marrow microenvironment and to develop better drugs to enhance platelet function.

Key Points

- Platelets are essential to promote hemostasis
- Platelet function is characterized by adhesion, activation, aggregation
- Platelet substitutes can be divided into erythrocyte derivatives, liposomal products, and nanoparticles
- To date, there has not been a viable platelet substitute

References

1. Ellingson KD, Sapianzo MRP, Haass KA, et al. Continue decline in blood collection and transfusion in the United States-2015. Transfusion. 2017;27:1588–98.
2. Wagner CL, Mascelli MA, Neblock DS, Weisman HF, Coller BS, Jordan RE. Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. Blood. 1996;88:907–14.
3. Coller BS, Springer KT, Beer JH, et al. Thromboerythrocytes. In vitro studies of potential autologous, semi-artificial alternative to platelet transfusions. J Clin Invest. 1992;89:546–55.
4. Chan LWG, White NJ, Pun SH. Synthetic strategies for engineering intravenous hemostats. Bioconjug Chem. 2015;26(7):1224–36.
5. Nishiya T, Kainoh M, Murata M, Handa M, Ikeda Y. Platelet interactions with liposomes carrying recombinant platelet membrane glycoproteins or fibrinogen: approach to platelet substitutes. Artif Cells Blood Substit Immobil Biotechnol. 2001;29:453–64.
6. Fisher TH, Merricks E, Bellinger DA, et al. Splenic clearance mechanisms of rehydrated, lyophilized platelets. Artif Cells Blood Substit Immobil Biotechnol. 2001;29(6):439–51.
7. Casals E, Galan AM, Escolar G, Gallardo M, Estelrich J. Physical stability of liposomes bearing hemostatic activity. Chem Phys Lipids. 2003;125(2):139–46.
8. Andrieux A, Hudry-Clergeon G, Ryckewart JJ, et al. Amino acid sequences in fibrinogen mediating its interaction with its platelet receptor, GPIIIbIIIa. J Biol Chem. 1989;264:9258–65.
9. Okamura Y, et al. Haemostatic effects of polymerized albumin particles carrying fibrinogen γ-chain dodecapeptide as platelet substitutes in severely thrombocytopenic rabbits. Transfus Med. 2008;18:158–66.
10. Coller BS. Interaction of normal, thrombasthenic, and Bernard-Soulier platelets with immobilized fibrinogen: defective platelet-fibrinogen interaction in thrombasthenia. Blood. 1980;55:169–78.
11. Yen RCK, Ho TWC, Blajchman MA. A new haemostatic agent: thrombospheres shorten the bleeding time in thrombocytopenic rabbits. Thromb Haemost. 1995;73:986–9.
12. Levi M, Friederich PW, Middleton S, de Groot PG, Wu YP, Harris R, Biemond BJ, Heijnen HF, Levin J, Cate JW. Fibrinogen-coated albumin microcapsules reduce bleeding in severely thrombocytopenic rabbits. Nat Med. 1999;5:107–11.
13. Okamura Y, Fukui Y, Kabata K, Suzuki H, Handa M, Ikeda Y, Takeoka S. Novel platelet substitutes: disk-shaped biodegradable nanosheets and their enhanced effects on platelet aggregation. Bioconjug Chem. 2009;20:1958–65.
14. Estcourt LJ, et al. Antifibrinolytics (lysine analogues) for the prevention of bleeding in people with haematological disorders. Cochrane Database Syst Rev. 2016;(3):CD009733.

15. Gernsheimer TB, Brown SP, Triulzi DJ, et al. Effects of tranexamic acid prophylaxis on bleeding outcomes in hematologic malignancy: the a-TREAT trial. Blood. 2020;136(Supplement 1):1–2.
16. Kristensen J, Killander A, Hippe E, Helleberg C, Ellegard J, Holm M, Kutti J, Mellqvist UH, Johansson JE, Glazer S, Hedner U. Clinical experience with recombinant factor VIIa in patients with thrombocytopenia. Haemostasis. 1996;26(Suppl. 1):159–64.
17. Wilde MI, Faulds D. Oprelvekin: a review of its pharmacology and therapeutic potential in chemotherapy-induced thrombocytopenia. BioDrugs. 1998;10(2):159–71.
18. Kaushansky K. Thrombopoietin: understanding and manipulating platelet production. Annu Rev Med. 1997;48:1–11.
19. Basser RL, O'Flaherty E, Green M, et al. Development of pancytopenia with neutralizing antibodies to thrombopoietin after multicycle chemotherapy supported by megakaryocyte growth and development factor. Blood. 2002;99:2599–602.
20. Cheloff AZ, Al-Samkari H. Avatrombopag for the treatment of immune thrombocytopenia and thrombocytopenia of liver disease. J Blood Med. 2019;10:313–21.

Plasma Substitutes

18

Christopher Ryan Hoffman, Alexander Huynh, and Henry Liu

Background

Plasma substitutes are widely used in practice for fluid resuscitation in the management of critically ill patients resulting from trauma, hemorrhage, sepsis, major surgery, and burns.

For decades, much has been debated on which is the ideal substitute regarding colloids vs crystalloids. This has been broadened further to include colloid vs colloid with the advent of synthetic colloids (hydroxyethyl starch, dextrans, gelatins) in addition to the natural colloid albumin. Clinicians are faced with the challenge of choosing a wide range of available products. Careful consideration of the products' pharmacokinetics, pharmacodynamics, and side effect profile should be incorporated [1].

Hypovolemia may be absolute (blood volume loss) or relative (blood volume redistribution). Either causes insufficient blood volume to maintain organ perfusion, vascular wall tension, venous return, and ultimately cardiac output leading to shock states [1]. In acutely ill patients, blood volume expansion is the recommended 1st line intervention in the "Surviving Sepsis" resuscitation guidelines for patients with sepsis or septic shock. This state is defined as a life-threatening organ dysfunction caused by dysregulated host response to infection [2]. This state of hypovolemia involves widespread distribution of fluids into the interstitial compartment as well as vasodilation. Other etiologies include increased loss of fluids from hemorrhage, diarrhea, vomiting, or decreased fluid intake. Intravascular volume is needed to transport nutrients, waste products, and oxygen. Failure to achieve this elicits numerous complications and eventual multiorgan dysfunction [3]. A reduced perfusion pressure due to greater than 30% loss of circulatory volume has been shown to lead to multiorgan failure and death [4].

Issues arise when choosing the ideal agent for fluid management. The ideal fluid should remain in the intravascular space for several hours and have a chemical composition similar to that of extracellular fluid. Constituents should be able to be metabolized and excreted by the body. The fluid should be safe, sterile, and not prone to organ toxicity [5]. The fluids can be classified into crystalloids and colloids. Crystalloids are further broken down by their tonicity and electrolyte composition. Common solutions include 3% normal saline, 0.9% normal saline, plasmalyte, lactated ringer (LR), and dextrose 5%. Colloids are high molecular weight substances in crystalloid solutions. They retain intravascular volume due to high oncotic pressure. Colloid solutions include albumin, hydroxyethyl starches (HES), dextran, and gelatin solutions (Table 18.1).

The recommendations for empiric replacement of fluid have evolved in the past, stemming from studies looking at fluid resuscitation in multiple patient settings [6]. In a 2001 landmark study looking at early goal-directed therapy (EGDT), it was found that prompt intravenous (IV) fluid administration was critical in improving inpatient mortality compared to a control group. Standard therapy consisted of arterial and central venous catheterization with a protocol targeting a CVP of 8–12 mmHg, mean arterial pressure (MAP) of 65 mmHg and a urine output of at least 0.5 mL/kg/h. The EDGT group included all elements of this standard but included a catheter measuring central venous saturation (SvO_2), protocolized administration of 500 mL of IV crystalloid every 30 minutes targeting CVP, SVO_2, and MAP goals [7]. More recently, three multicenter trials have called into question the benefit of EGDT. These trials were the ProCess, ARISE and ProMISe trials which showed that EGDT showed no difference than usual care [8–10]. Regardless of the study comparison, it is still recommended that patients in sepsis receive early administration of IV fluid to correct hypovolemia, improve blood pressure, and optimize tissue perfusion. The optimal amount, rate, and end point is still unknown and is continuously evolving [11].

C. R. Hoffman · A. Huynh
Thomas Jefferson University Hospital, Philadelphia, PA, USA

H. Liu (✉)
Anesthesiology & Critical Care, University of Pennsylvania, Philadelphia, PA, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_18

Table 18.1 Common volume replacement solutions

	Solution	Source	Concentration	Pros	Cons
Crystalloid	Normal saline	Electrolyte solution	0.9%	Inexpensive Widely available Mild hypertonicity preferable in TBI	Hyperchloremic metabolic acidosis Limited intravascular expansion Third spacing Fluid overload
	Balanced solutions (e.g., Plasmalyte, Lactated Ringer's)	Electrolyte solution		More physiologically balanced/closer to serum osmolarity	Potential accumulation of electrolytes/lactate Suboptimal in neurosurgery
	Hypertonic saline	Electrolyte solution	2%, 3%, 5%, 7%	Transient hemodynamic improvement compared to other crystalloid Heightened oncotic activity/fluid retention Reduces ICP Increase T-cell/interleukin proliferation	Rapid administration may cause pontine myelinolysis Excess administration can exacerbate volume overload Long-term infusion may require central venous access
	Hypotonic saline	Electrolyte solution	0.45%, 0.3%, 0.18%	Treats hypertonic dehydration/free fluid loss	Rapid administration may cause cerebral edema Excess administration can worsen electrolyte deficit
Natural Colloid	Albumin	Human	4%, 5%, 25%	Potential anti-inflammatory effects, studies vary Potential benefit in sepsis setting	Expensive Not universally available No statistically significant benefit over crystalloid in multiple settings (trauma, TBI)
Synthetic Colloid	Hydroxyethyl Starches	Potato/Maize	6%, 10%	More cost effective than other colloids Higher maximum allowable doses than other colloids Decreased incidence of new onset heart failure in ICU setting	Hypersensitivity reactions Coagulopathy Increased incidence of hepatic failure and renal failure necessitation replacement therapy in ICU setting
	Dextrans	*Leuconostoc mesenteroides*	10% dextran 40, 6% dextran 70	Improved viscosity with similar hemodilution Decreased immune mediated leukocyte/endothelial interaction Improves microcirculatory flow in ECMO/CPB settings	Hypersensitivity reactions Coagulopathy Potential interference with cross matching Renal dysfunction
	Gelatins	Bovine	3.5%, 4%	Cost-effective Rapid excretion, short half-life Safer maximum allowable dose, if any Less risk of renal dysfunction than other colloids	Hypersensitivity reactions Coagulopathy Circulatory dysfunction Renal dysfunction still evident

TBI traumatic brain injury, *ICP* intracranial pressure, *ECMO* extracorporeal membrane oxygenation, *CPB* cardiopulmonary bypass

Crystalloids

Isotonic Solutions

Crystalloids are compositions of fluid and electrolyte ions in varying proportions that determine tonicity. Crystalloids first appeared during the cholera pandemic in 1832 and consisted of sodium, chloride, and bicarbonate in water [12]. Historically, 0.9% sodium chloride has been the most commonly administered IV fluid [13]. However, the use of more balanced solutions closer to human plasma electrolyte composition have grown in popularity [14]. Balanced crystalloids are solutions in which chloride anions are replaced with bicarbonate or buffers to reduced acid-base balance perturbation [15]. Isotonic crystalloids can be categorized into two basic categories which include sodium chloride and physiologically balanced solutions. The balanced solutions include lactated ringers and plasmalyte.

Normal saline: The term normal saline is derived from the Dutch physiologist Hartog Hamburger in 1882 who suggested 0.9% concentration of salt as found in human blood [12]. Normal saline contains sodium and chloride in equal concentrations of 154 mmol/L, isotonic with extracellular fluid. The osmolarity of the solution is approximately 308 mOsm/L. The levels of sodium and chloride exceed normal physiological levels in the extracellular fluid [16]. This high chloride load has been associated with the development of hyperchloremic acidosis. Additionally, adverse effects such as renal impairment, immune dysfunction, and inability to recover from severe illness have been described [17, 18].

Lactated ringers' solution: Lactated ringers (LR) solution (also known as Hartmann's or sodium lactate solution) is a mixture containing sodium chloride, sodium lactate, potassium chloride and calcium chloride in water [19]. The solution has an osmolarity of 273 mOsm/L, making it hypotonic to serum. LR should subsequently be avoided in patients with cerebral edema as it can worsen edema. Another common fear related to LR is the risk of causing hyperkalemia or lactic acidosis due to its contents. LR contains a 4 mEq/L potassium concentration. However, due to the large volume of distribution and equilibration between the intracellular and extracellular compartments, increases in potassium are not typically seen even in renal failure patients [20, 21]. Many would argue that the risk of acidosis caused by normal saline administration would increase the risk of hyperkalemia greater than administering LR. LR has also been thought to raise serum lactate levels due to the 28 mmol/L of sodium lactate. Lactate is metabolized by the liver and kidney and is thought to transiently raise serum lactate levels in periods of large administration. In trials looking at 1 l and 30 mL/kg LR boluses vs normal saline in septic patients, no differences were found in mean lactate levels after administration [22, 23]. LR also contains calcium, which makes it incompatible with blood product administration as it can chelate with citrate and develop clot.

Plasmalyte and the congeners: Plasmalyte was first made available in 1982 and engineered to be similar to plasma. It contains sodium, potassium, chloride, and magnesium at levels similar to plasma. The osmolality of plasmalyte is approximately 291 mOsm/kg which falls within the normal physiological range of 280–296 mOsm/kg. One of the main differences between plasmalyte and LR is the addition of magnesium and omission of calcium. The addition of magnesium to plasmalyte should be considered when given to patients at risk for hypermagnesemia. Although plasmalyte contains magnesium, it is not indicated for the treatment of hypomagnesemia. Similar to LR, plasmalyte contains 5 mmol/L of potassium and should be used cautiously in patients at risk for hyperkalemia. There have been no published cases seen in medical literature for plasmalyte hypersensitivity reactions [24]. Interestingly, patients have reported to falsely test positive for galactomannan antigen after receiving plasmalyte infusion. Galactomannan is a marker for pulmonary aspergillosis in immunocompromised patients [25].

Numerous studies have been conducted over the years looking to determine the ideal balanced crystalloid. These studies range from preclinical research to comparison studies between saline and balanced crystalloids in observational and controlled trials among healthy volunteers, acute illness, operating room, and critically ill adults. Among healthy volunteers receiving 2 L of either saline or plasmalyte over 1 hour, it was shown that saline decreased renal artery velocity, cortical perfusion, and urine output, and increased extravascular fluid accumulation compared to plasmalyte [26].

Observational studies comparing balanced crystalloids to saline in the resuscitation of patients in septic shock have been described [27, 28]. A retrospective analysis of 100,000 patients from an electronic record database meeting SIRs criteria showed patients receiving larger chloride loads had increased risk of death (odds ratio, 1.09; 95% confidence interval) [27]. Another study found that in a propensity matched analysis of 6000 adults, patients receiving balanced crystalloids were associated with a 3.2% lower absolute risk of in-hospital mortality [28]. Many studies have looked at the intraoperative difference among different crystalloids. The LICRA (Limiting IV Chloride to Reduce AKI) study is the largest trial to date comparing normal saline to balanced crystalloids. This trial was a single, cluster, double- crossover trial comparing low-chloride solutions with high chloride solutions among 1126 adults going cardiac surgery at one academic center. The incidence of AKI was not significantly different between the two groups [29]. The Saline vs Lactated Ringers (SOLAR) trial showed no difference in postoperative complications in patients undergoing orthopedic and colorectal surgery [30].

Numerous studies also address the critically ill. The SPLIT trial (0.9% Saline versus Plasmalyte 148 for ICU fluid Therapy) compared 2278 patients admitted to ICU. The patients were predominantly admitted after cardiovascular surgery. They showed a 0.87 relative risk of in-hospital mortality with balanced crystalloids versus saline [31]. The SALT (isotonic Solution Administration Logistical Testing) compared LR with 0.9% saline among 974 adults admitted from the ED to the ICU. This trial showed a 0.91 odds ratio of 30 day in-hospital mortality with balanced crystalloids versus saline (95% CI 0.64–1.30). There was also decreased incidence of death, new renal replacement therapy, or renal dysfunction with balanced crystalloids versus saline [32].

The SMART trial (isotonic Solutions and Major Adverse Renal events Trial) examined 15,802 patients from five ICUs. The primary endpoint was major adverse kidney events within 30 days (MAKE30), composite of death, and persistent renal dysfunction. This trial showed an increased

MAKE30 among the saline group, and an absolute risk reduction in MAKE of 3.7% (0.6–6.9%) and in-house mortality of 4.2% (−7.9–16.4%) with balanced solution [33]. As one might expect, the trials and studies examining the ideal isotonic agent are vast. The decision to administer a crystalloid depends on numerous factors including cost efficacy, clinical judgement, and data driven decision making.

Hypotonic and Hypertonic Solutions

Different solutions based on their osmolality have been developed and used in medical practice, including both hypotonic and hypertonic solutions. Although not primarily used as plasma expanding solutions in resuscitation, these solutions can substitute plasma and have varying medical applications. Hypotonic solutions commonly used include dextrose in water and 0.45% saline. Hypotonic solutions have been used in the pediatric population since the mid 1950s. This study detailed maintenance fluid requirements in children and found that the standard maintenance rate for sodium was approximately 4 mmol/kg of sodium per day [34]. Hypotonic fluids which contain approximately 30–50 mmol/L of sodium is consistent with this requirement and led to its use in the pediatric population. However, significant side effects of hypotonic solutions have led to concerns for widespread use. These include development of hyponatremia and cerebral edema. The passive osmotic shift from the hypotonic plasma to the brain can lead to significant neurological morbidity. One Cochrane review showed a marked lower risk of developing hyponatremia in those receiving isotonic solutions versus hypotonic solutions (17% vs 34%) [35]. Serum sodium levels are lowered in patients receiving hypotonic solutions versus isotonic solutions [36].

Hypertonic solutions come in different formulations including 3–7.5% saline. These solutions are composed of sodium and chloride dissolved in water at concentrations much higher than normal blood serum levels. The FDA approved uses of hypertonic solutions include correcting hyponatremia and increased intracranial pressure. The high concentration of NaCl compared to plasma causes an osmotic gradient that drives fluid into the intravascular space. This causes an increase in mean arterial pressure (MAP), stroke volume (SC) and cardiac output (CO) when compared to other isotonic fluids [37]. Additionally, it has been found that less volume of hypertonic saline is required to achieve similar plasma volumes of normal saline [38]. Its use in elevated ICP settings relate to its ability to increase serum osmolarity, allowing fluid from the extravascular space to enter the intravascular space. Studies show that 3% hypertonic saline decreases ICP similar to 20% mannitol [39]. In patients with severe hyponatremia, hypertonic saline can be used for efficient correction. The optimal regimen is variable and restrictive. Authors recommend only up to a 6–12 mEq/L increase in sodium in the first 24 hours, and 18 mEq/L in 48 hours should be performed due to risk for central pontine myelinolysis [40]. It is also noted that central venous access is the preferred route of administration.

Colloids

Natural Protein Colloid – Albumin

Albumin is a plasma protein naturally occurring in human serum. Developments in blood fractionation led to the isolation, purification, and subsequent marketing of albumin solutions as a resuscitative agent (Fig. 18.1). At a molecular weight of 66.5 kDa, it is derived from pooled human plasma then heated and sterilized by ultrafiltration. Purified protein fractions yield 83–96% albumin, with the remainder isolated being various globulins. FDA package insert indications for albumin include hypovolemia, ascites, hypoalbuminemia including from burns, acute nephrosis, acute respiratory distress syndrome, and cardiopulmonary bypass [42]. Albumin is typically available in 4%, 5%, 20%, and 25% concentrations, with clinical indications for higher strengths focusing on severe liver disease and shock states [43]. Despite its lengthy history on the market, it remains expensive to produce and distribute, limiting its use worldwide. The SAFE (Saline versus Albumin Fluid Evaluation) study estimated that one 500 cc bottle of 4% albumin cost €61.80, or $75.62 [44]. Low concentration albumin is relatively iso-oncotic, while high concentration is substantially hyper-oncotic. 500 mL of 5% albumin expands plasma volume by ~500–

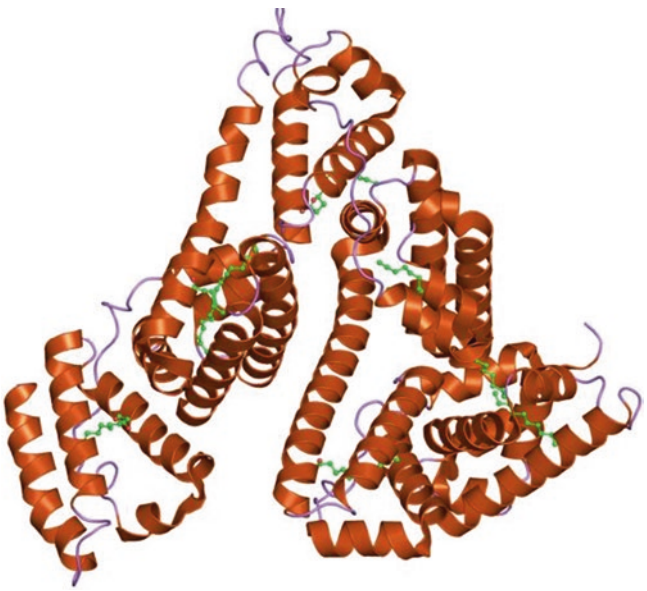


Fig. 18.1 Cartoon representation of molecular structure of serum albumin [41]

750 mL, while 100 mL of 25% albumin expands plasma volume by ~450 mL. Albumin has an intravascular residence time of about 4 hours [45]. This is an approximation, as plasma volume expansion may be dependent on rate of administration and extent of systemic disease (e.g., cirrhosis) [46, 47].

Multiple studies over time have attempted to determine albumin's role in optimizing organ perfusion and tissue oxygenation. Investigation into the matter heightened after a Cochrane meta-analysis of 30 randomized controlled trials including 1419 participants concluded that those receiving albumin instead of crystalloid in the setting of hypovolemia, burns, or hypoalbuminemia had a higher pooled risk of death (relative risk 1.68, number needed to harm = 17) [48]. The SAFE study subsequently followed 6997 ICU patients receiving 4% albumin or crystalloids for 28 days. Results showed no significant difference in pooled mortality or the development of new organ failure. Of note, to achieve no differences in mean arterial pressure or heart rate, the ratio of albumin to saline administered was 1:1.4. The trial did report that pathology-specific outcomes differed. Those in the group being treated for traumatic brain injury did associate with increased mortality (relative risk 1.63), albeit at 2 years post intervention. Those in the group being treated for hypoalbuminemia did not exhibit statistically significant difference. Those in the group being treated for severe sepsis were associated with decreased mortality in the 28-day study window (relative risk 0.71) [44]. Extrapolation of data from these findings estimated cost efficacy for those treated with albumin specifically in the setting of severe sepsis. Real world admissions and discharges in this patient population combined with estimated life expectancy yielded costs per life saved and per year saved were €6037 ($7385) and €617 ($755), respectively [49]. Aforementioned studies were restricted to adult populations. Evidence has shown no statistically significant difference in mortality in the pediatric population with impaired perfusion receiving bolus resuscitation at 48 hours, though there was an increase in death at 48 hours in both groups [50].

Investigations clarifying albumin's role in inflammatory processes and endothelial cell activation is largely restricted to in vitro study with limited conclusions. Treatment of bovine cells to human albumin yielded mild anti-inflammatory properties when compared to hydroxyethyl starch [51]. Rat models pose that albumin treatment may prevent lung injury in the setting of hemorrhagic shock when compared to crystalloid administration [52]. Conversely, human umbilical venous tissue models have demonstrated albumin mediated increases in endothelial cell adhesion molecules (e.g., e-selectin, ICAM-1, VCAM-1), potentially worsening critically ill patients [53]. In the clinical realm, there has been less beneficial effect seen, though potentially a lack of deleterious effect. Plasma concentrations of soluble adhesion molecules (e.g. sELAM-1, sICAM-1, sVCAM-1, sGMP-140) remained at baseline concentrations in the setting of trauma, versus increases when compared to hydroxyethyl starch administration. The exact reason for this is unclear, but authors pose that the consistent inflammatory mediator concentration could have been due to it being unaffected just as potentially as it being continued or worsened [54].

Potential adverse effects are not limited to the debate over inflammatory processes. The principal concern is with regard to hypersensitivity reactions. The reported incidence of any reaction is 1 in 6600, with severe reactions occurring in 1 in 30,000 patients [55]. History or heightened concern for allergy is the principal contraindication according to the FDA. The other chief concern is its administration in the setting of resuscitation (severe anemia, cardiac failure) in the setting of increased intravascular volume, due to the potential for impending fluid overload [56]. Albumin should be used with caution in the settings where capillary or barrier tissue has been violated, such as injuries to lung tissue or the blood brain barrier. The elimination of filtering barriers normally allowing for transudative fluid shifts may now result in albumin/oncotic mediated sequestration of fluid. Albumin leaking into interstitium in these settings may exacerbate pulmonary compromise or cerebral edema. Evidence suggests albumin mediated coagulopathy is another complication to consider when weighing risks and benefits of administration. In vitro reports detail dose dependent attenuation of histone-induced platelet aggregation, as well as a return to normal function when albumin is depleted from plasma [57]. Other studies pose that albumin increases the formation of anti-aggregatory prostaglandin D_2 and E_2 from cyclic endoperoxides [58, 59].

Synthetic Colloids

Hydroxyethyl starches (HES) are hydrolyzed derivatives of amylopectin, a highly branched cornstarch. Glucose molecules are hydroxyethylated at one or more of six carbon positions (Fig. 18.2). Variations of HES depend on the degree of molar substitution (the proportion of glucose units on the starch molecule modified with hydroxyethyl units) and ratio of CS-C6 substitutions. These changes result in higher molecular weights and altered degradation/excretion rates [61]. Due to this wide variety, HES compounds are listed with particular nomenclature (Table 18.2). For example, the product Hespan is 6% HES 450/0.7 in normal saline. The first number (6%) denotes the concentration of the solution, the second number (450) denotes the mean molecular weight in kDs, and the third number (0.7) denotes the molar substitution (in this case, approximately 7 hydroxyethyl groups for every 10 glucose units) [62]. Typically 6% HES solutions are considered iso-oncotic, meaning that they can replace other colloids (e.g. albumin, blood product) at a 1:1 ratio. 10%

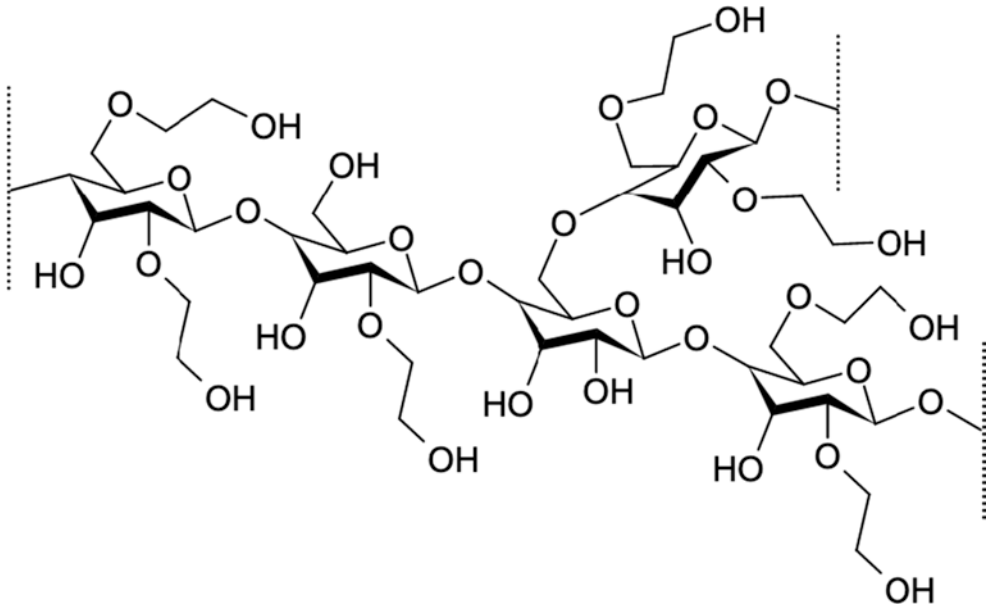


Fig. 18.2 Structure of hydroxyethyl starch [60]

Table 18.2 Hydroxyethyl starch preparations

HES Preparation	Concentration	Mean molecular weight (kDa)	Molar substitution	C2/C6 ratio	Osmolarity (mOsmol/kg)	Maximum daily dosage (mL/kg)
Hextend	6% hetastarch in balanced solution	670	0.75	5:1	307	20
Hespan	6% hetastarch in 0.9% saline	600	0.75	5:1	309	20
Hestar	6% pentastarch in 0.9% saline	200	0.5	5:1	308	33
Haes-Steril	6% tetrastarch in 0.9% saline	200	0.43–0.55	5:1	308–310	33
	10% tetrastarch in 0.9% saline					20
Venofundin	6% tetrastarch in 0.9% saline	130	0.42	6:1	309	50
Tetraspan	6% tetrastarch in balanced solution	130	0.42	6:1	296	50
	10% tetrastarch in balanced solution					33
Tetrahes	6% tetrastarch in 0.9% saline	130	0.4	>8:1	308	50
VetStarch	6% tetrastarch in 0.9% saline	130	0.4	9:1	308	50
Voluven	6% tetrastarch in 0.9% saline	130	0.4	9:1	308	50
Volulyte (Voluven in solution instead of saline)	6% tetrastarch in balanced solution	130	0.4	9:1	286.5	50

HES solutions are hyperoncotic, determined to expand volume by approximately 145%.

Molecular weights are commonly designated by the mean molecular weight, though some sources organize starches by number average molecular weight. This calculation is simply the total weight of the polymer divided by the total number of molecules. The mean molecular weight is therefore influenced by larger molecules in the polymer and reports larger numbers. Ratios of the two calculations delineates the degree of nonuniformity in each polymer. When a polymer solution is administered, oncotic activity is determined by the weight of individual particles, not the total averaged weight. If a nonuniform solution is introduced the smaller, easily broken-down particles will clear while the heavily weighted particles remain. Furthermore, breakdown of heavily weighted particles may still exhibit oncotic activity until they are broken down further and cleared. The difference in ratios may not have a clinical effect, as the smaller cleared particles are offset by the larger particles and their relatively larger breakdown products. Ultimately, varying molecular weights have a lesser impact of clinical outcome than molar substitutions. This is demonstrated in products with similar molecular weights but differing molar substitutions [63].

Hydroxyethyl group substitutions to the glucose polymer increase water solubility and inhibit enzymatic breakdown. This is typically reported as the number of hydroxyethyl groups per glucose subunit. There can be multiple residues per glucose subunit. Therefore, the number may be inflated when compared to the degree of substitution, a figure calculated from the total number of substituted groups divided by the total number of glucose residues. The degree of substitution determines nomencla-

ture for certain products. Solutions with a molar substitution of 0.7 are referred to as hetastarches, 0.6 as hexastarches, 0.5 as pentastarches, and 0.4 as tetrastarches [64]. Newer generation starches possess reduced substitution coefficients, lacking the risk for persistent retention in the plasma. Plasma clearance of tetrastarch is 20 times more rapid than pentastarches and hetastarches [65].

One key pharmacokinetic property not established in the HES nomenclature is the C2/C6 ratio. As previously mentioned, hydroxyethyl groups can be attached to multiple sites on a glucose subunit. They are most commonly positioned at the C2 and C6 carbons of the glucose molecule. Enzymatic hydrolysis of HES products occurs via serum α-amylase. Amylase can access substrates more effectively in HES products with higher proportions of C6 substitutions than C2 substitutions. Therefore, the higher the C2/C6 ratio, the more resilient the compound is to enzymatic breakdown. Plasma concentration and subsequent oncotic activity has been shown to increase as the C2/C6 ratio increases, with a "high ratio" exceeding 8:1 [66, 67].

HES solutions are the most widely used and studied synthetic colloids, commonly administered and reported in military and ICU settings. The primary advantage in these settings is that HES solutions are more cost effective than other colloids, including albumin. Similar to other synthetic colloids, its sequestration limits the maximum allowable dose. One advantage however is that the maximum volume (around 50 mL/kg) is greater than other synthetic colloids. It has been demonstrated that HES solutions may be preferred in sepsis settings. HES solutions exhibit anti-inflammatory properties and preserve gastrointestinal microvasculature in patients treated for bacteremia [68]. HES solutions' adverse effects have been also commonly reported. One of the more common wide scale reviews of HES administration is the CHEST study (Crystalloid versus Hydroxyethyl Starch Trial), where 7000 ICU patients receiving HES solutions were investigated. The group found no difference in 90-day mortality between HES and saline administration in the setting of sepsis, trauma, or traumatic brain injury. A decreased incidence of new onset heart failure was demonstrated, though alongside increased incidence of hepatic failure and renal failure necessitation replacement therapy [69]. HES solutions are associated with higher incidences of anaphylactoid reactions, when compared to other colloids [70]. While newer, more rapidly degraded HES solutions exhibit mitigated effect, all HES solutions affect coagulation and platelet function. Specific mechanisms are not identified, but there is up to an 80% subsequent decrease in Factor VIII, von Willebrand factor, and platelet count. This can manifest in administration less than maximum allowable doses, yielding delayed coagulation studies and increased bleeding complications [71, 72]. HES sequestration varies based on the generation of the solution and dose dependence. It has been identified in liver, muscle, spleen, placenta, intestine, and skin. Radio-labeling has detected significant and varying quantities of HES in tissue over 50 days after its administration [73]. Aside from limiting maximum doses, this sequestration often presents with extensive pruritus.

Dextrans (10% Dextran 40, 6% Dextran 70)

Dextran consists of glucose molecules derived from *Leuconostoc mesenteroides* polymerized into chains of high molecular weight polysaccharides. The compound is manufactured into varying molecular weights, and as such each commercially available product is named after its target weight. The most common sizes marketed are dextran 40 and dextran 70, with 40 kD and 70 kD molecular weights, respectively. These formulations exhibit high water binding capacity. One gram of dextran 40 retains 30 mL of water and 1 g of dextran 70 retains about 20–25 mL of water, precipitating a 100–150% increased volume [74]. The compounds benefit from longer intravascular residence times than albumin. The majority of dextran 40 is cleared within 5 hours and the majority of dextran 70 is cleared within 6–8 hours, though as much as 40% of dextran can remain in circulation up to 12 hours. Both polymers are almost entirely cleared via the kidneys. Trace amounts can enter the interstitium and gastrointestinal tract [75].

In addition to gross volume expansion, dextrans' role in resuscitation has been in the setting of ischemia, post-ischemia, and reperfusion injury. Animal studies show that dextran solutions markedly decrease blood viscosity with the same degree of hemodilution when compared to other plasma substitutes [76]. Theoretically the improved viscosity reduces the sticking interaction of activated leukocytes with endothelial cell membrane. Limiting leukocyte activation at the cell membrane decreases harmful released intermediates. Authors also posit the involvement of dextrans binding to adhesion molecules. Animal models exhibit this, with decreased immune mediated leukocyte/endothelial interaction after induced ischemia and hemorrhagic shock [77–79]. Dextran has subsequently been relied upon to improve microcirculatory flow in microsurgical re-implantation, extracorporeal circulation, and cardiopulmonary bypass.

Dextran is not widely utilized largely related to its side effect profile. Dextrans reactive antibodies cause more severe anaphylactic reactions than any of the other synthetic colloids. This incidence can be lowered by pretreating with 20 mL single dose containing 3 g of dextran 1. Antibodies are believed to bind to dextran 1 instead and form inactive complexes. However as this is also a dextran, the pretreatment also carries hypersensitivity risk. Pretreatment does not completely eliminate the potential for anaphylaxis to dextran 40 or 70. Dextrans promote coagulopathy, inducing a von Willebrand-like condition. Von Willebrand factor and subsequent vWf:Factor VIII activity is attenuated, decreasing

platelet adhesiveness. Increased fibrinolysis has also been demonstrated. These effects are dose dependent, with greater effect in larger molecular weight dextrans. As a result, there is a maximal dosage recommendation of 1.5 g dextran/kg body weight/day to prevent serious bleeding and increased transfusion requirement [80]. Dextrans have been reported to interfere with cross matching. Peripheral smears show RBC clumping from dextran mediated false agglutination of the red cells. This activity can be resolved by washing the patient's red cells several times with saline [81, 82]. Renal dysfunction associated with dextran administration is well documented. Acute renal failure and exacerbation of chronic failure have both been demonstrated, though it is more commonly associated with preexisting renal disease, oliguria, hemodynamic instability, advanced age, and long-term dextran administration. The likely mechanism is accumulating dextran molecules in the renal tubules causing tubular plugging, swelling, and vacuolization [83].

Gelatins

Gelatins are a group of products formed from collagen hydrolysis. They are typically derived from dissolving bovine connective tissue and forming jellies once cooled. Despite their animal origin, gelatin products are considered sterile, preservative-free, and shelf stable for 3 years when stored at less than 30 °C. Early solutions (circa 1915) had higher molecular weights than other plasma expanders, with some products weighing 100 kD and higher. These compounds exhibited marked oncotic effect but carried increased risk related to its viscosity. Its storage was complicated by solidification when stored at low temperatures [84].

Various solutions have subsequently been developed, with varying molecular weights and chemical modifications. Succinylated or fluid modified gelatins (Gelofusine, Plasmagel, Plasmion) contain 20 kD polypeptides modified by the addition of succinic acid. It is a preservative-free 4% solution with electrolytes (Na^+ 154, K^+ 0.4, Ca^{++} 0.4 and Cl^- 120 mmol/L), its composition considered useful during aggressive fluid resuscitation. The low chloride may be useful in the setting of hyperchloremic metabolic acidosis associated with excess normal saline administration, and the low calcium content allowing for compatibility with concomitant blood transfusion [84]. Urea-crosslinked gelatins (Polygeline, Hemacel) contain 12–15 kD polypeptides crosslinked with hexamethyl di-isocyanate to form compounds with weights ranging from 5–50 kD, with an average of 24.5 kD. It is a preservative-free 3.5% solution with electrolytes (Na^+ 145, K^+ 5.1, Ca^{++} 6.25 & Cl^- 145 mmol/L). It is noted for its comparatively elevated potassium and chloride concentrations [84, 85]. Oxypolygelatin (Gelifundol) is considered one of the initial developed compounds in the second-generation gelatins, modified by oxidation via hydrogen peroxide. It is a 5.5% solution with electrolytes (Na^+ 130 & HCO_3^- 30, Cl^- 100 mmol/L) [86, 87]. It is often grouped with new generation gelatins as a topic but has not been heavily utilized or cited since the 1990s.

Gelatin administration has been shown to expand volume 70–80% greater than crystalloid solutions, with expansion and hemodiluting abilities comparable to HES [88]. The compounds are also rapidly renally excreted, with less than 3% metabolized. They demonstrate less risk to renal impairment than other plasma expanders. 24 hours after administration, gelatin is reported to be found approximately 71% in the urine, 16% extravascular and 13% in plasma. Unlike other colloids, its rapid transit eliminates the need for upper limits of administration. It is markedly cheaper to produce than other colloids as well. The cost efficacy and lack of volume restriction may mitigate the overall increased product required due to its short half-life (approximately 2.5 hours) [84, 89]. As a result, clinical indications have included hypovolemia secondary to acute hemorrhage, normovolemic hemodilution, extracorporeal circulation, and volume preloading prior to neuraxial anesthesia [89, 90].

Gelatin administration is associated with increased anaphylactic and anaphylactoid reactions. They are associated with a higher incidence of such reactions when compared to other synthetic colloids. Meta-analysis of its use demonstrated a 1.15 risk ratio for mortality and a 3.01 risk ratio for anaphylaxis [70, 91]. Early studies implied gelatins had little effect on coagulation other than hemodilution. Evidence has grown that gelatins demonstrate more effect, though the clinical outcome is unclear. Hypocoagulability has been demonstrated with increased prothrombin time and international normalized ratios, as well as increased reaction time on thromboelastography [92]. Gelatin administration has been shown to inhibit ristocetin-induced platelet agglutination and create varied effects on GP IIb–IIIa expression [93, 94]. Circulatory dysfunction is evident as gelatin administration is associated with increased transcapillary filtration with rebound to baseline after its administration is discontinued [95]. Meta-analysis of gelatin administration has demonstrated circulatory dysfunction associated with increased plasma renin activity, albumin excretion, and alpha/beta-microglobinuria. This translates to a 1.35 risk ratio for acute kidney injury and/or renal failure [61, 91].

Summary

The ideal resuscitation fluid would markedly increase plasma volume, exhibit similar iso-oncotic and isotonic composition to extracellular fluid, and limit third-spacing extravasation. It would produce no adverse effects during or after its administration. Its sequestration would be trace, and its clearance and excretion would be complete in relatively short duration. Its elimination would not rely upon optimally functioning

organ systems. It would be easily produced, cost-effective, and readily available with a long shelf life without special storage requirements. These parameters are not achieved by any product utilized today. Yet the need for optimal resuscitation in trauma and intensive care arenas has resulted in decades of research and development. Many products have entered the market, with a range of mechanisms, indications, and adverse effects. Despite expectations embellished by biochemical and pharmacodynamic properties, colloid solutions do not exert a clear benefit over crystalloid solutions with regard to hemodynamic outcomes. This lack of statistical superiority, combined with a diverse side effect profile, largely leads to synthetic colloid solutions being left out of general resuscitation guidelines and recommendations. Albumin administration is often restricted by its expense and limited statistical benefit over crystalloid solution, though some scenarios exist where it can be beneficial. Balanced salt solutions have become the common practical solution for most clinical scenarios, though they are not without their own safety and efficacy concerns. Ultimately, not every product is available in every location or scenario. Thus, administration of any particular product is often dependent on regional availability, clinical preference, and cost efficacy.

Key Points

- An ideal plasma substitute should hypothetically increase plasma volume and demonstrate similar composition to extracellular fluid. It would be free of adverse effects, cost-effective, with a long shelf-life.
- Conventional crystalloid solutions are inexpensive, widely available, and well-known. They are often considered the standard plasma substitute, though they can sequester into interstitium, exacerbate fluid overload, and accumulate electrolyte.
- Purified human serum albumin is a natural colloid solution that can effectively expand volume via osmotic pressure, depending on the concentration given. It is expensive with limited availability. Its administration is advantageous in sepsis but likely harmful in burns and traumatic brain injury. Other clinical settings report little statistical benefit, limiting widespread use.
- Hydroxyethyl starches include a wide variety of polymers with osmotic activity dependent on their molecular weight and moiety substitutions. Recent compounds are cost-effective and have demonstrated potential benefits in the setting of heart failure. Wider use is limited by adverse effects, including hypersensitivity reactions, coagulopathy, and hepatorenal failure.
- Dextrans are glucose molecules polymerized into chains of high molecular weight polysaccharides commonly produced in two molecular weights, dextran 40 and dextran 70. They exhibit dramatic water retention with improved viscosity, leukocyte interaction, and microcirculatory flow. Wider use is limited by adverse effects, including hypersensitivity reactions, coagulopathy, and renal failure.
- Gelatins are derived from dissolving bovine connective tissue and forming jellies once cooled. Newer generation compounds exhibit cost-efficacy, stable shelf life, and dramatic water retention. Their clearance allows for safer maximum allowable doses. Wider use is limited by adverse effects, including hypersensitivity reactions, coagulopathy, and renal failure.

References

1. Perner A, Cecconi M, Cronhjort M, Darmon M, Jakob SM, et al. Expert statement for the management of hypovolemia in sepsis. Intensive Care Med. 2018;44(6):791–8.
2. Rhodes A, Evans LE, Alhazzani W, Levy MM, Antonelli M, et al. Surviving sepsis campaign: international guidelines for management of sepsis and septic shock: 2016. Intensive Care Med. 2017;43(3):304–77.
3. Mullen JV, Wise R, Vermeulen G, Monnen PJ, Malbrain M. Assessment of hypovolaemia in the critically ill. Anesthesiol Intensive Ther. 2018;50(2):150–9.
4. Rossaint R, Bouillon B, Cerny V, Coats TJ, Duranteau J, et al. The European guideline on management of major bleeding and coagulopathy following trauma: fourth edition. Crit Care. 2016;20:100.
5. MacDonald N, Pearse RM. Are we close to the ideal intravenous fluid? Br J Anaesth. 2017;119:63–71.
6. Angus DC, van der Poll T. Severe sepsis and septic shock. N Engl J Med. 2013;369:840–51.
7. Rivers E, Nguyen B, Havsted S, Ressler J, Muzzin A, et al. Early goal-directed therapy in the treatment of severe sepsis and septic shock. N Engl J Med. 2001;345:1368–77.
8. Yealy DM, Kellum JA, Huang DT, Barnato AE, Weissfeld LA, et al. A randomized trial of protocol-based care for early septic shock. N Engl J Med. 2014;370:1683–93.
9. Peake SL, Delaney A, Bailey M, Bellomo R, Cameron PA, et al. Goal-directed resuscitation for patients with early septic shock. N Engl J Med. 2014;371:1496–506.
10. Mouncey PR, Osborn TM, Power GS, Harrison DA, Sadique MZ, et al. Trial of early, goal-directed resuscitation for septic shock. N Engl J Med. 2015;372:1301–11.
11. Semler MW, Rice TW. Sepsis resuscitation: fluid choice and dose. Clin Chest Med. 2016;37:241–50.
12. Awad S, Alllison SP, Lobo DN. The history of 0.9% saline. Clin Nutr. 2008;27(2):179–88.
13. Finfer S, Liu B, Taylor C, Bellomo R, Billot L, et al. Resuscitation fluid in critically ill adults: an international cross sectional study in 391 intensive care units. Crit Care. 2010;14(5):R185.
14. Hammond NE, Taylor C, Finfer S, Machado FR, An Y, et al. Patterns of intravenous fluid resuscitation use in adult intensive care patients between 2007 and 2014: an international cross-sectional study. PLoS One. 2017;12(5):e0176292.
15. Morgan TJ. The ideal crystalloid – what is "balanced?". Curr Opin Crit Care. 2013;19:299–307.
16. Lobo DN. Intravenous 0.9% saline and general surgical patients: a problem, not a solution. Ann Surg. 2012;255:830–2.

17. Kellum JA, Song M, Li J. Science review: extracellular acidosis and the immune response: clinical and physiologic implications. Crit Care. 2004;8(5):331–6.
18. Shaw AD, Bagshaw SM, Goldstein SL, Scherer LA, Duan M, et al. Major complications, mortality, and resource utilization after open abdominal surgery: 0.9% saline compared to plasma-lyte. Ann Surg. 2012;255:821–9.
19. Iqbal U, Anwar H, Scribani M. Ringer's lactate versus normal saline in acute pancreatitis: a systemic review and meta-analysis. J Dig Dis. 2018;19(6):335–41.
20. Khajavi MR, Etezadi F, Moharari RS, Imani F, Meysamie AP, et al. Effects of normal saline vs. lactated ringer's during renal transplantation. Ren Fail. 2008;30(5):535–9.
21. Modi MP, Vora KS, Parikh GP, Shah VR. A comparative study of impact of infusion of ringer's lactate solution versus normal saline on acid-base balance and serum electrolytes during live related renal transplantation. Saudi J Kidney Dis Transpl. 2012;23(1):135–7.
22. Didwania A, Miller J, Kassel D, Jackson EV Jr, Chernow B. Effect of intravenous lactated Ringer's solution infusion on the circulating lactate concentration: part 3. Results of a prospective, randomised, double-blind, placebo- controlled trial. Crit Care Med. 1997;25:1851–4.
23. Zitek T, Skaggs ZD, Rahbar A, Patel J, Khan M. Does intravenous lactated ringers solution raise serum lactate? J Emerg Med. 2018;55(3):313–8.
24. Weinberg L, Collins N, Van Mourik K, Tan C, Bellomo R. Plasma-Lyte 148: a clinical review. World K Crit Care Med. 2016;5(4):235–50.
25. Petraitiene R, Petraitis V, Witt JR 3rd, Durkin MM, Bacher JD, et al. Galactctomannan antigenemia after infusion of gluconate-containing Plasma-lyte. J Clin Microbio. 2011;40:4330–2.
26. Chowdhury AH, Cox EF, Francis ST, Lobo DN. A randomized controlled, double blind crossover study on the effects of 2-L infusions of saline and Plasma-Lyte 148 on renal blood flow and renal cortical tissue perfusion in healthy volunteers. Ann Surg. 2012;256:18–24.
27. Shaw AD, Raghunathan K, Peyerl FW, Munson SH, Paluszkiewicz SM, et al. Association between intravenous chloride load during resuscitation and in-hospital mortality among patients with SIRS. Intensive Care Med. 2014;40:1897–905.
28. Raghunathan K, Shaw A, Nathanson, Stürmer T, Brookhart A, et al. Association between the choice of IV crystalloid and in-hospital mortality among critically ill adults with sepsis. Crit Care Med. 2014;42:1585–91.
29. McIlroy D, Murphy D, Kasza K, Bhatia D, Wutzlhofer L, et al. Effects of restricting perioperative use of intravenous chloride on kidney injury in patients undergoing cardiac surgery: the LICRA pragmatic controlled trial. Intensive Care Med. 2017;42:795–806.
30. Maheshwari K, Turan A, Makarova N, Ma C, Esa WAS, et al. Saline versus lactated ringer's solution: the saline or lactated ringer's (SOLAR) trial. Anesthesiology. 2020;132(4):614–24.
31. Young P, Bailey M, Beasley R, Henderson S, Mackle D, et al. Effect of a buffered crystalloid solution vs saline on acute kidney injury among patients in the intensive care unit: the SPLIT randomized clinical trial. JAMA. 2015;314:1701–10.
32. Semler MW, Wanderer JP, Ehrenfeld JM, Stollings JL, Self WH, et al. Balanced crystalloids versus saline in the intensive care unit: the SALT randomized trial. Am J Respir Crit Care Med. 2017;195:1362–72.
33. Semler MW, Self WH, Wanderer JP, Ehrenfeld JM, Wang L, et al. Balanced crystalloids versus saline in critically ill adults. N Engl J Med. 2018;378:829–39.
34. Holliday MA, Segar WE. The maintenance need for water in parenteral fluid therapy. Pediatrics. 1957;19(5):823–32.
35. McNab S, Ware R, Neville K, Choong K, Coulthard MG, et al. Isotonic versus hypotonic solutions for maintenance intravenous fluid administration in children. Cochrane Database Syst Rev. 2014;(12):CD009457.
36. Friedman J, Beck C, DeGroot J, Geary DF, Sklansky DJ, et al. Comparison of isotonic and hypotonic intravenous maintenance fluids: a randomized clinical trial. JAMA Pediatr. 2015;169(5):455–1.
37. Pfortmueller CA, Schefold JC. Hypertonic saline in critical illness – a systematic review. J Crit Care. 2017;42:168–77.
38. Joseph B, Aziz H, Snell M, Pandit V, Hays D, et al. The physiological effects of hyperosmolar resuscitation: 5% vs 3% hypertonic saline. Am J Surg. 2014;208(5):697–702.
39. Sokhal N, Rath GP, Chaturvedi A, Singh M, Dash HH. Comparison of 20% mannitol and 3% hypertonic saline on intracranial pressure and systemic hemodynamics. J Clin Neurosci. 2017;42:148–54.
40. Adrogue HJ, Madias NE. Hyponatremia. N Engl J Med. 2000;342(21):1581–9.
41. Swaminathan J. Cartoon representation of the molecular structure of protein registered with 1e7f code, 2009. Public domain, Wikimedia Commons Accessed 23 Feb 2021.
42. 5% human albumin [package insert]. Bio Products Laboratory Ltd., Durham. https://www.fda.gov/media/113691/download. Accessed 18 Dec 2020.
43. Lira A, Pinsky MR. Choices in fluid type and volume during resuscitation: impact on patient outcomes. Ann Intensive Care. 2014;4:38.
44. Finfer S, Bellomo R, Boyce N, French J, Myburgh J, et al. A comparison of albumin and saline for fluid resuscitation in the intensive care unit. N Engl J Med. 2004;350:2247–56.
45. McChaon R, Hardman J. Pharmacology of plasma expanders. Anaesth Intensive Care Med. 2007;8(2):79–81.
46. Statkevicius S, Bonnevier J, Fisher J, Bark BP, Larsson E, et al. Albumin infusion rate and plasma volume expansion: a randomized clinical trial in postoperative patients after major surgery. Crit Care. 2019;23(1):191.
47. Brinch K, Møller S, Bendtsen F, Becker U, Henriksen JH. Plasma volume expansion by albumin in cirrhosis. Relation to blood volume distribution, arterial compliance and severity of disease. J Hepatol. 2003;39(1):24–31.
48. Cochrane Injuries Group Albumin Reviewers. Human albumin administration in critically ill patients: systematic review of randomised controlled trials. BMJ. 1998;317(7153):235–40.
49. Guidet B, Mosqueda GJ, Priol G, Aegerter P. The COASST study: cost-effectiveness of albumin in severe sepsis and septic shock. J Crit Care. 2007;22(3):197–203.
50. Maitland K, Kiguli S, Opoka RO, Engoru C, Olupot-Olupot P, et al. Mortality after fluid bolus in African children with shock. N Engl J Med. 2011;364:2483–95.
51. Lang JD, Figueroa M, Chumley P, Aslan M, Hurt J. Albumin and hydroxyethyl starch modulate oxidative inflammatory injury to vascular endothelium. Anesthesiology. 2004;100:51–8.
52. Zhang H, Voglis S, Kim CH, Slutsky AS. Effects of albumin and Ringer's lactate on production of lung cytokines and hydrogen peroxide after resuscitated hemorrhage and endotoxemia in rats. Crit Care Med. 2003;31:1515–22.
53. Nohé B, Dieterich HJ, Eichner M, Unertl K. Certain batches of albumin solutions influence the expression of endothelial cell adhesion molecules. Intensive Care Med. 1999;25:1381–5.
54. Boldt J, Heesen M, Padberg W, Martin K, Hempelmann G. The influence of volume therapy and pentoxifylline infusion on circulating adhesion molecules in trauma patients. Anaesthesia. 1996;51(6):529–35.
55. McClelland DB. ABC of transfusion: human albumin solutions. BMJ. 1990;300:35–7.
56. Laxenaire MC, Charpentier C, Feldman L. Anaphylactoid reactions to colloid plasma substitutes: incidence, risk factors, mechanisms. A french multicenter prospective study. Ann Fr Anesth Reanim. 1994;13(3):301–10.

57. Lam FW, Cruz MA, Leung HE, Parikh KS, Smith CW, et al. Histone induced platelet aggregation is inhibited by normal albumin. Thromb Res. 2013;132(1):69–76.
58. Gresele P, Deckmyn H, Huybrechts E, Vermylen J. Serum albumin enhances the impairment of platelet aggregation with thromboxane synthase inhibition by increasing the formation of prostaglandin D2. Biochem Pharmacol. 1984;33(13):2083–8.
59. Frederick ED, Hausburg MA, Thomas GW, Rael LT, Brody E, et al. The low molecular weight fraction of human serum albumin upregulates COX2, prostaglandin E2, and prostaglandin D2 under inflammatory conditions in osteoarthritic knee synovial fibroblasts. Biochem Biophys Rep. 2016;8:68–74.
60. Mattern R. Structure of hydroxyethyl starch, 2009. Public domain, Wikimedia Commons. Accessed 23 Feb 2021.
61. Davidson IJ. Renal impact of fluid management with colloids: a comparative review. Eur J Anaesthesiol. 2006;23(9):721–38.
62. Westphal M, James MFM, Kozek-Langenecker S, Stocker R, Guidet B, Van Aken H, Warner MA. Hydroxyethyl starches: different products – different effects. Anesthesiology. 2009;111:187–202.
63. Asskali F, Förster HL. The accumulation of different substituted hydroxyethyl starches (HES) following repeated infusions in healthy volunteers. Anasthaesiol Intens Notfall Schmerz. 1999;34:537–41.
64. Kozek-Langenecker SA. Effects of hydroxyethyl starch solutions on hemostasis. Anesthesiology. 2005;103:654–60.
65. Jungheinrich C, Sauermann W, Bepperling F, Vogt NH. Volume efficacy and reduced influence on measures of coagulation using hydroxyethyl starch 130/0.4 (6%) with an optimised in vivo molecular weight in orthopedic surgery: a randomised, double-blind study. Drugs R&D. 2004;5:1–9.
66. Gandhi SD, Weiskopf RB, Jungheinrich C, Koorn R, Miller D, Shangraw RE, Prough DS, Baus D, Bepperling F, Warltier DC. Volume replacement therapy during major orthopedic surgery using voluven® (hydroxyethyl starch 130/0.4) or hetastarch. Anesthesiology. 2007;106:1120–7.
67. Jungheinrich C, Neff TA. Pharmacokinetics of hydroxyethyl starch. Clin Pharmacokinet. 2005;44:681–99.
68. Schäper J, Ahmed R, Schäfer T, Elster A, Enigk F, Habazettl H, et al. Volume therapy with colloid solutions preserves intestinal microvascular perfusion in endotoxaemia. Resuscitation. 2008;76(1):120–8.
69. Myburgh JA, Finfer S, Bellomo R, Billot L, Cass A, Gattas D, et al. Hydroxyethyl starch or saline for fluid resuscitation in intensive care. N Engl J Med. 2012;367:1901–11.
70. Barron ME, Wilkes MM, Navickis RJ. A systematic review of the comparative safety of colloids. Arch Surg. 2004;139(5):552–63.
71. de Jonge E, Levi M, Buller R, Berends F, Kesecioglu J. Decreased circulating levels of von Willebrand factor after intravenous administration of a rapidly degradable hydroxyethyl starch (HES 200/0.5/6) in healthy human subjects. Intensive Care Med. 2001;27:1825–9.
72. Linder P, Ickx B. The effects of colloid solutions on hemostasis. Can J Anesth. 2006;53:30–s39.
73. Leuschner J, Opitz J, Winkler A, Scharpf R, Bepperling F. Tissue storage of 14C-labelled hydroxyethyl starch (HES) 130/0.4 and HES 200/0.5 after repeated intravenous administration to rats. Drugs RD. 2003;4:331–8.
74. Martino P, editor. The ICU book. Colloid and crystalloid resuscitation. 3rd edition. Philadelphia: Churchill Livingstone; 2007. p. 233–54..
75. Mitra S, Khandelwal P. Are all colloids same? How to select the right colloid? Indian J Anaesth. 2009;53(5):592–607.
76. Menger MD, Sack FU, Hammersen F, Messmer K. Tissue oxygenation after prolonged ischemia in skeletal muscle: therapeutic effect of prophylactic isovolemic hemodilution. Adv Exp Med Biol. 1989;248:387–95.
77. Nolte D, Illner A, Menger MD. Reduction of postischemic leukocyte-endothelial interaction by Dextran 70 and not by hydroxyethyl starch 200/0.62. Int J Microcirc Clin Exp. 1992;11:210–6.
78. Schmand J, Ayala A, Chaudry IH. Effects of trauma, duration of hypotension, and resuscitation regimen on cellular immunity after hemorrhagic shock. Crit Care Med. 1994;22:1076–83.
79. Bauer M, Marzi I, Ziegenfuss T, Seeck G, Buhren V, et al. Comparative effects of crystalloid and small volume hypertonic hyperoncotic fluid resuscitation on hepatic microcirculation after hemorrhagic shock. Circ Shock. 1993;40:187–93.
80. Kaye AD, Kucera IJ. Intravascular fluid and electrolyte physiology. In: Miller RD, editor. Miller's anesthesia. 6th ed. Philadelphia: Churchill Livingstone; 2005. p. 1763–98.
81. Nearman HS, Herman ML. Toxic effects of colloids in the intensive care unit. Crit Care Clin. 1991;7(3):713–23.
82. Arora K, Stocks C, Bryan E, Harrison C. ABO discrepancy caused by iron dextran. Am J Clin Pathol. 2012;138(suppl_2):A315.
83. Dubois MJ, Vincent JL. Colloid fluids. In: Hahn RG, Prough DS, Svensen CH, editors. Perioperative fluid therapy. 1st ed. New York: Wiley; 2007. p. 153–611.
84. Roberts J, Nightingale P. Properties and use of gelatins. In: Webb AR, editor. Therapeutics. Germany: Braun; 2003. p. 45–52.
85. Saddler JM, Horsey PJ. The new generation gelatins: a review of their history, manufacture, and properties. Anaesthesia. 1987;42:998–1004.
86. Campbell DH, Koepfli JB. The preparation and properties of a modified gelatin (oxypolygelatin) as an oncotic substitute for serum albumin. Technical Information Pilot, Issues 18900–21399, 1952 Reference Abstracts. p. 3804.
87. Daniels FH, Nedev ND, Cataldo T, Leonard EF, Cortell S. The use of polyelectrolytes as osmotic agents for peritoneal dialysis. Kidney Int. 1988;33:925–9.
88. Awad S, Dharmavaram S, Wearn CS, Dube MG, Lobo DN. Effects of an intraoperative infusion of 4% succinylated gelatine [Gelofusine[R]] and 6% hydroxyethyl starch [Voluven[R]] on blood volume. Br J Anaesth. 2012;109(2):168–76.
89. Goodnough L, Monk T. Autologous transfusion. In: Miller RD, editor. Miller's anesthesia. 6th ed. Philadelphia; 2005. p. 1831–43.
90. Baraka AS, Taha SK, Ghabach MB, Sibaii AA, Nader AM. Intravascular administration of polymerized gelatin versus isotonic saline for prevention of spinal-induced hypotension. Anesth Analg. 1994;78(2):301–5.
91. Moeller C, Fleischmann C, Thomas-Rueddel D, Vlasakov V, Rochwerg B, Theurer P, Gattinoni L, Reinhart K, Hartog CS. How safe is gelatin? A systematic review and meta-analysis of gelatin-containing plasma expanders vs crystalloids and albumin. J Crit Care. 2016;35:75–83.
92. Sawhney C, Subramanian A, Kaur M, Anjum A, Albert V, Soni KD, Kumar A. Assessment of hemostatic changes after crystalloid and colloid fluid preloading in trauma patients using standard coagulation parameters and thromboelastography. Saudi J Anaesth. 2013;7(1):48–56.
93. Thaler U, Deusch E, Kozek-Langenecker SA. In vitro effects of gelatin solutions on platelet function: a comparison with hydroxyethyl starch solutions. Anaesthesia. 2005;60(6):554–9.
94. Evans PA, Heptinstall S, Crowhurst EC, Davies T, Glenn JR, et al. Prospective double-blind randomized study of the effects of four intravenous fluids on platelet function and hemostasis in elective hip surgery. J Thromb Haemost. 2003;1(10):2140–8.
95. Holbeck S, Grände PO. Effects on capillary fluid permeability and fluid exchange of albumin, dextran, gelatin, and hydroxyethyl starch in cat skeletal muscle. Crit Care Med. 2000;28(4):1089–95.

Part III

Products in Development

Soluble Nanobiotherapeutics with Enhancements of All Three Major Red Blood Cell Functions

19

Thomas Ming Swi Chang

Introduction

General

Artificial Red blood cells (RBC) containing stroma-free hemoglobin [1] was prepared to have some of the 3 major RBC functions: (1) transport oxygen (2) remove oxygen radicals and (3) Transport carbon dioxide CO2. We also crosslinked stroma-free hemoglobin using glutaraldehyde [2] to form crosslinked stroma-free hemoglobin [3]. Most people thought that artificial RBC was a simple matter that could be quickly developed for clinical use when needed. Thus, only the other areas of artificial cells were extensively developed around the world [4].

Hemoglobin Based Oxygen Carrier

When H.I.V. came unexpectedly in the 1980s there was no blood substitute, and many patients were infected with H.I.V. contaminated donor blood. The initial urgency with H.I.V. contaminated donor RBC led researchers to concentrate on simple oxygen carriers without the other RBC functions. Thus, Biopure used glutaraldehyde to crosslink ultrapure bovine hemoglobin to form polyhemoglobin (PolyHb) with only one RBC function, oxygen carrier. This has been approved in South Africa and Russian to avoid the use of H.I.V. contaminated donor blood [5]. In another clinical trial using a different polyhemoglobin [6] there was concern regarding a small increase in nonfatal myocardial infarction. It was thought that this could be due to the lack of the antioxidant function of the polyhemoglbin [7].

Oxygen Carrier with Antioxidant Activity

Thus, a soluble complex of nanobiotherapeutic consisting of hemoglobin and antioxidant enzymes was formed by crosslinking hemoglobin (Hb) with two RBC antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) to form Poly-[Hb-SOD-CAT] [7] Later, conjugated hemoglobin containing synthetic antioxidants (PNPH) [8] have also been prepared. Both of these can remove oxygen radicals and prevent ischemia-reperfusion injury. Other approaches include those of Simoni et al., Rousselot et al., Jia & Alayash as discussed in detail elsewhere in this book.

Oxygen and Carbon Dioxide Carriers with Antioxidant Activity

Do we need all 3 RBC functions in some conditions as in sustained severe hemorrhagic shock? Sim et al. [9] showed in animal study that increase in intracellular pCO2 is related to increase fatality in severe hemorrhagic shock animal. They also showed that intracellular pCO2 was not the same as blood pCO2. Tronstad et al. [10] showed that increase intracellular pCO2 was correlated with myocardial ischemia. We therefore prepared soluble nanobiotherapeutic with enhancement of all 3 RBC functions by adding carbonic anhydrase (CA) to the antioxidant enzymes to form a novel soluble nanobiotechnological complex Poly-[Hb-SOD-CAT-CA] [11–15]. By increasing the concentrations of the enzymes, we could enhance RBC functions for oxygen radical removal and CO_2 transport [11–15].

The text and figures of this paper is from papers by the same author from Artificial Cells, Nanomedicine and Biotechnology with copy right permission.

T. M. S. Chang (✉)
Artificial Cells and Organs Research Centre, Departments of Physiology, Medicine and Biomedical Engineering, Faculty of Medicine, McGill University, Montreal, QC, Canada
e-mail: Thomas.chang@mcgill.ca; http://www.artcell.mcgill.ca

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_19

Soluble Nanobiotherapeutics with Enhancement of All 3 RBC Functions: Poly-[Hb- SOD-CAT-CA]

Method

We crosslinked hemoglobin (Hb), superoxide dismutase (SOD), catalase (CAT) and carbonic anhydrase (CA) into a soluble Poly-[Hb-SOD-CAT-CA] nanobiotechnological complex [11]. It not only had all 3 RBC functions, but it could also have enhancement of all 3 RBC functions by increasing the concentrations of RBC enzymes in the complex. Thus, we can enhance the enzyme activity of Poly-[Hb-SOD-CAT-CA] [11, 12]. For example, enzymes (units/dl) for Poly- [Hb-SOD-CAT-CA] (SOD 195K; CAT 3500K, CA 1300K) could be higher than those for Poly-SFHb: (SOD 70K,CAT 470K,CA 950K. With our later study of extraction of RBC enzymes, we were able to adjust the enzyme concentration to 2, 4 and 6 times that of red blood cells [16]

Result in a 2.3 Blood Loss 90 min Sustained Hemorrhagic Shock Rat Model

We studied this in rats with 90 min hemorrhagic shock at 30 mm Hg mean arterial blood pressure (MAP) by removing 2/3 of blood volume [11]. Mean blood pressures recovered after the infusion of all test infusions except for lactated Ringer (Fig. 19.1). However, analysis of the data shows that Poly-[Hb-SOD-CAT-CA] with enhanced enzymes was significantly ($p < 0.05$) superior to blood in lowering of the elevated tissue pCO2 (Fig. 19.1); recovery of the elevated ST (Fig. 19.2); lower troponin levels, lowering of elevated lactate, histology of the intestine (Fig. 19.2), kidney and heart [11]. PolySFHb is the crosslinking of stroma-free hemoglobin that contains hemoglobin and RBC enzymes at the concentration normally present in the RBC. It is less effective than Poly-[Hb-SOD-CAT-CA] but shows the same effectiveness as RBC and superior to PolyHb (Fig. 19.2) [11].

Striking changes are observed in the histology of the intestine (Fig. 19.2) [11]. When reperfused with the animal's own blood, there is some detachment of the epithelium from the villi suggesting some tissue injuries, but the gland architecture is still intact (Fig. 19.2). The injury in the PolyHb group (Fig. 19.2) showed injured villi and some damage to the glands but most of the glands still retain their structure. Poly-[Hb-SOD-CAT-CA] (Fig. 19.2) show intact mucosal structure with no obvious injuries. Histology of the heart also showed no injury when compared to blood, PolySFHb or PolyHb (Fig. 19.2).

Effects of 4 Weekly Toploading Followed by 30% Exchange: Safety and Immunological Effects [14]

General

We analyzed the effects of 1/10 blood volume toploading per week for 4 weeks followed by a 30% blood exchange [12]. We followed the well-being, growth, biochemistry, histology and also the important question of the immunological properties of this nanobiotechnological complex [12]. Using very sensitive specific test for antibodies to bovine hemoglobin and bovine enzymes, there was no increase in specific antibodies and there was no change in blood pressure before and after each of the 4-weekly injection. It would appear that the large amount of hemoglobin nanoencapsulate the small amount of enzymes and thus separated them from immunological reactions (Fig. 19.3).

Result

The long term safety and immunological effects of bovine poly-[hemoglobin-catalase-superoxide dismutase-carbonic anhydrase] in rats were studied by four-weekly 5% blood volume top-loading infusions followed by 30% blood volume exchange transfusion [12]. There is no significant differences in growth, biochemistry and blood pressure between the control group receiving lactated ringer solution and those receiving the bovine poly-[hemoglobin-catalase-superoxide dismutase- carbonic anhydrase] There is no significant change in mean arterial pressures (MAP) before and after each weekly top-loading infusion. After both the 4-weekly top-loading and the 30% exchange transfusions, the following safety and immune response evaluations are carried out. These include general studies on Ouchterlony double diffusion, total IgG and IgM, and complement activation.

This is followed by quantitative measurements of specific antibodies against each of the following bovine components: Hb, CAT, SOD and CA in bovine poly-[hemoglobin-catalase-superoxide dismutase-carbonic anhydrase]. After the four-weekly top-loading, each rat received a challenge of 30% blood volume exchange transfusion. The MAP, histamine and tryptase levels are tested before and after the 30% exchange transfusion. There are no anaphylactic reactions as shown by the MAP or histamine and tryptase. The results showed no safety problem nor adverse immune responses. All the rats survived when followed for 1 week after the 30% exchange transfusion.

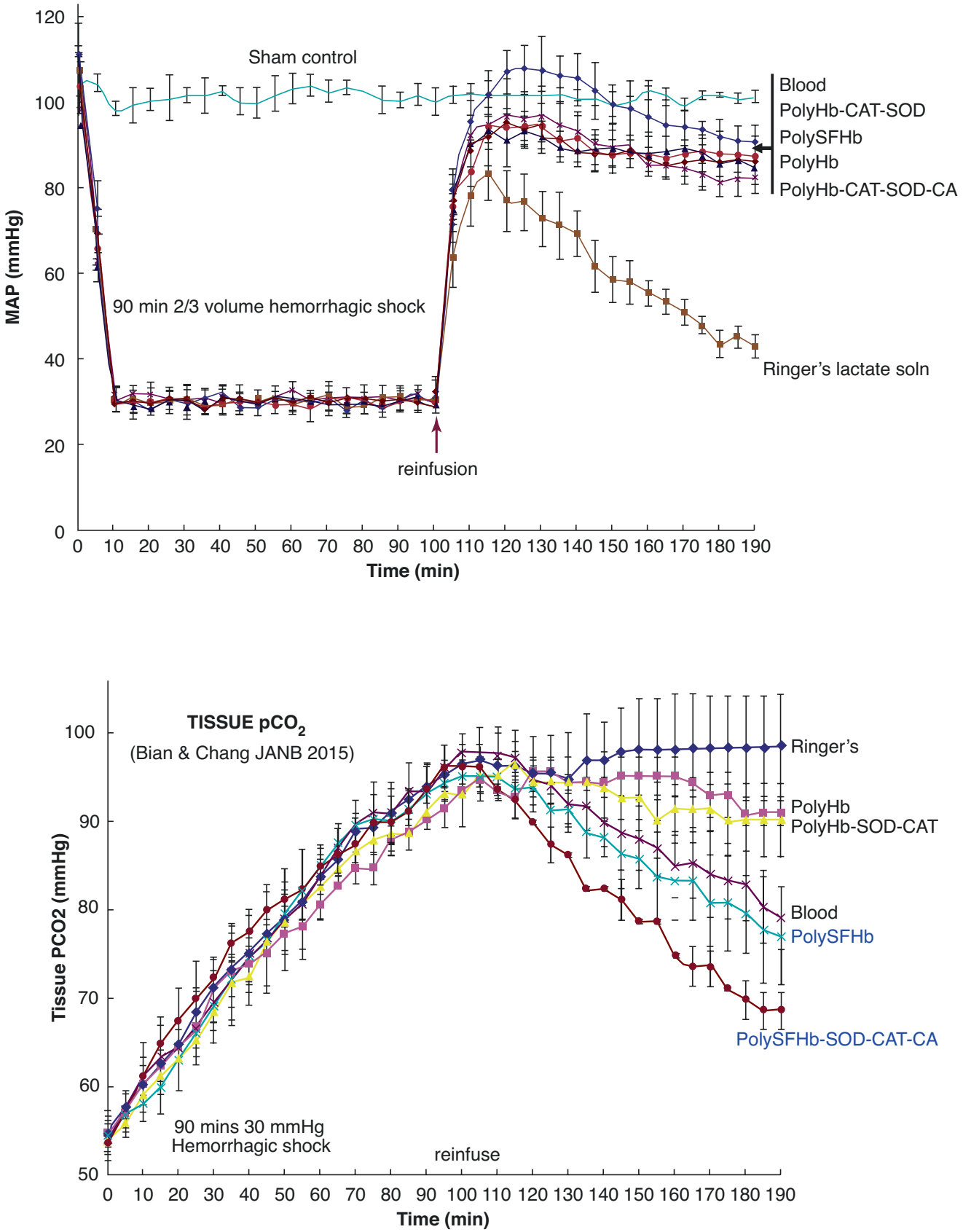
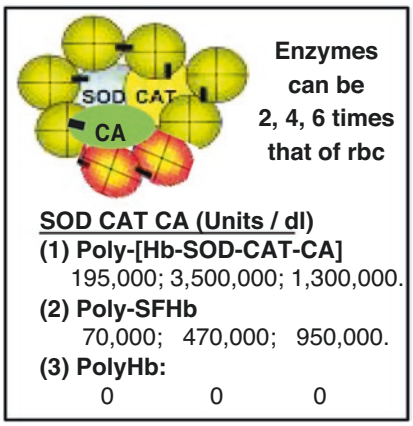
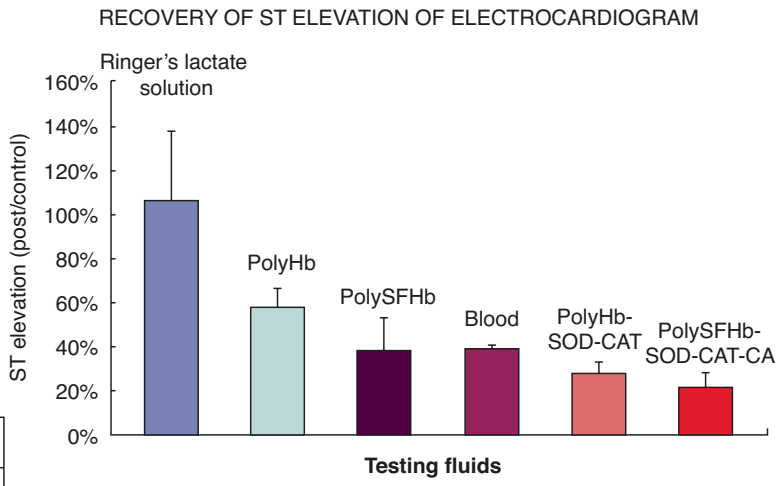


Fig. 19.1 ***Upper***: Removal of 2/3 blood volume followed by 90 min sustained hemorrhagic shock. Effects of different infusions on Mean Arterial Blood Pressure, ***Lower***: Effect of 90 min sustained hemorrhagic shock on intracellular pCO2 and effects of different transfusion fluids. (From Bian & Chang [11] with copyright permission)



Pathology report

	Heart	Intestine
Ringers lactated solution	Sarcoplasmic condensation; contraction bands	Damaged villi as well as gland
PolyHb	Band contraction	Injured villi and mild injured gland
Blood	Sarcoplasmic condensation and eosinophilic changes	Injured villi but intact gland
PolySFHb	Eosinophilic changes	No damage
PolySFHb-SOD-CAT	No injury signals	No damage
PolyHb-SOD-CAT-CA	No injury signals	No damage



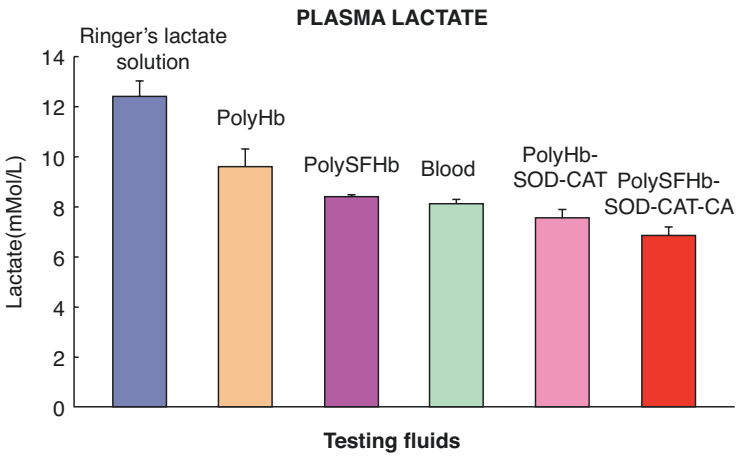
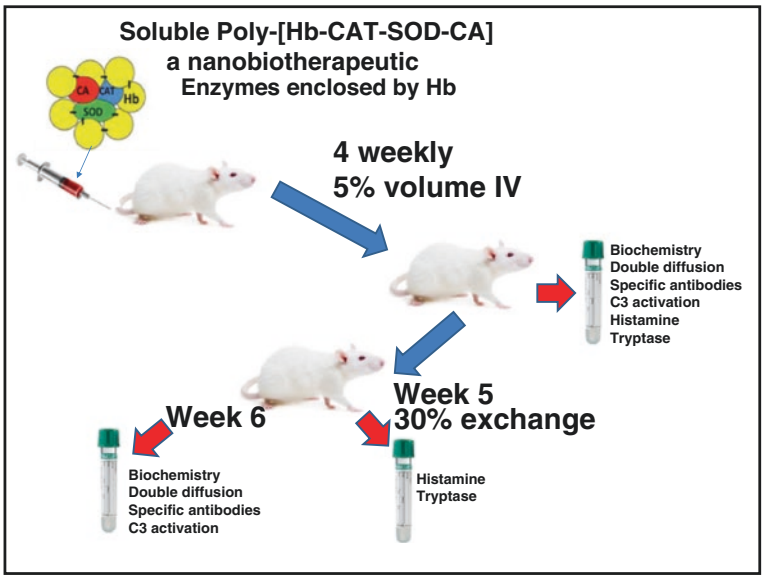


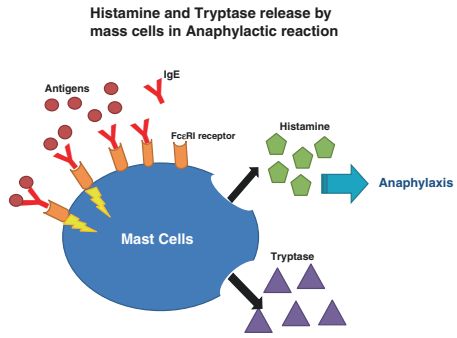
Fig. 19.2 ***Upper left:*** Enzyme concentrations in different systems ***Upper right:*** Effects of different infusions on the recovery of the heart as measured by the recovery of ST elevation. ***Lower right:*** Plasma lactate. PolyHb-SOD-CAT-CA reduced the plasma lactate level from 18% 2.3 mM/L to 6.9% 0.3 mM/L. It was significantly ($p < 0.05$) more effective than lactated Ringer's solution, PolyHb, blood, PolySFHb and PolyHb-SOD-CAT. ***Lower left:*** Pathology report on the histology of the heart and Intestine. (From Bian & Chang [11] with copyright permission)

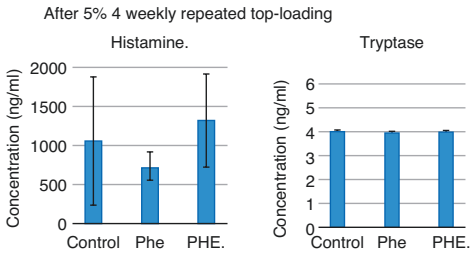
Test for Anaphylactic Reaction

Release of histamine by mast cells is responsible for anaphylactic reaction (Fig. 19.3). However, histamine is unstable, and measurement of plasma histamine is not accurate. Tryptase is also released by the mast cell in anaphylactic reaction. It is more stable and as a result the measurement of plasma tryptase is more accurate.

To investigate more quantitatively whether there are any anaphylactic reactions after the larger challenge of 30% blood volume, histamine and tryptase levels are tested before and 30 min after infusion of samples (LR, PHe or PHE) [12]. There was no significant differences in histamine and tryptase analysis when comparing the LR control group and the bovine PolySFHb (PHe) and poly- [Hb-CAT-SOD-CA] (PHE) groups [12] (Fig. 19.3). Histamine and tryptase levels were tested before and 30 min after 30% blood volume exchange transfusion of LR (control), PHe or PHE. The histamine levels in LR control group before and after infusion were 1058.62 ± 825.06 ng/ml and 695.47 ± 384.02 ng/ml. The histamine levels in PHe group before and after infusion were730.37 ± 182.28 ng/ml and 992.30 ± 334.91 ng/ml. The







- No difference in growth, biochemistry and blood pressure compared to control
- After the 4-weekly top-loading and the 30% exchange transfusions there was no significant differences compared to control in: Ouchterlony double diffusion, total IgG, IgM, and complement activation. specific antibodies against bovine Hb, CAT, SOD and CA.
- The challenge of 30% blood volume exchange transfusion did not result in anaphylactic reactions as shown by the MAP, histamine and tryptase.
- All the rats survived when followed for one week after

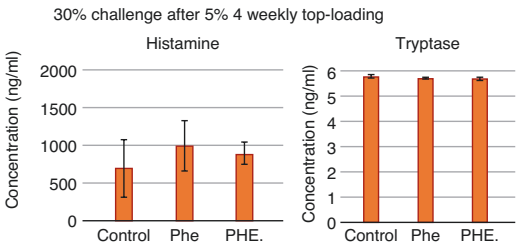


Fig. 19.3 ***Upper left:*** The rats are divided into 3 groups each receives 4 weekly infusion of one of the following samples: lactated ringer's solution (LR) as control group, bovine PolySFHb (Phe) and bovine poly-[Hb-CAT-SOD-CA] (PHE) with enhanced enzyme activity. This is followed on week 5 by a 30% exchange infusion. ***Lower left:*** Summary of safety and immunological studies. ***Right:*** In anaphylactic reaction both Histamine and Tryptase are released from the mast cells. Phe:PolySFHb. PHE: Poly-[Hb-CAT-SOD-CA]. There is no significant changes in the histamine or tryptase levels between the control group and the PHe and PHE groups. (From Guo & Chang [14] with copyright permission)

histamine levels in PHE group before and after infusion ere1323.64 ± 598.83 ng/ml and 882.72 ± 156.77 ng/ml. The p-value by one-way ANOVA among the three groups is 0.62 (>0.05). The tryptase levels before infusion in LR, PHe and PHE groups were 4.04 ± 0.03 ng/ml, 4.01 ± 0.01 ng/ml, and 4.04 ± 0.03 ng/ml; while after infusion, the values werr 5.79 ± 0.06 ng/ml for the LR control group, 5.73 ± 0.02 ng/ml for PHe, and 5.71 ± 0.05 ng/ml for PHE. By comparing the LR control groups and the blood substitutes groups, the p-value by one-way ANOVA was 0.51 (>0.05) before infusion, and the p-value of after infusion is 0.21 (>0.05). Thus, there was no significant changes in the histamine or tryptase levels between the control group and the PHe and PHE groups.

Storage Stability and Pasteurization

STABILITY AT DIFFERENT TEMPERATURES

Donor Red Blood Cells

Maximal time of storage allowed

20-25C	4C
1 day?	42 days

Poly-[Hb-SOD-CAT-CA] solution

T1/2 of enzyme activities:

	20-25C	4C
CAT	172 days	380 days
SOD	92 days	198 days
CA	51 days	231 days

Poly-[Hb-SOD-CAT-CA] freezedried:

Day 320% enzyme activity

	20-25C	4C
CAT	73%(day 320)	85%(day 320)
SOD	67%(day 320)	76%(day 320)
CA	73%(day 320)	85%(day320)

Unlike RBC or the solution form, the lyophilized form can be heat pasteurized at 70 °C for 2 hours to retain good enzyme activities of CA 97 ± 4%, SOD 100 ± 2.5% and CAT 63.8 ± 4% [13]. Adding more CAT in crosslinking can maintain the same enzyme ratio after pasteurization. Further investigation is needed to study the potential use of pasteurization of the lyophilized preparation as an additional step to the preparative procedure that involves crosslinking with glutaraldehyde and ultrafiltration that can inactivate or remove infective agents. FDA has already approved the sterilization method used for PolyHb.

Costs and Source of Enzymes

Purified enzymes from commercial sources are extremely expensive. Our research resulted in a novel method to simultaneously extract SOD, CAT and CA from the same sample of RBC [14].

This avoided the need for expensive commercial enzymes thus allowing this to be cost effective for future large-scale production of a nanobiotechnological Poly-[Hb-SOD-CAT-CA] with enhancement of all 3 red blood cell functions. The best concentration of phosphate buffer was analyzed and established resulting in good recovery of CAT, SOD and CA after extraction [14].

Different concentrations of the extracted enzymes could be used to enhance the activity of Poly-[Hb-SOD-CAT-CA] to 2, 4 or 6 times that of RBC. When conditions only require Poly-[Hb-SOD-CAT-CA] with the same concentration of enzymes as RBC, then the content of RBC can be easily use without the need for enzyme extraction.

This novel extraction method [14] will also allow us to extract the needed enzymes from any RBC source including discarded or contaminated donor RBC, post stem cell extracted human placental RBCs (cord RBCs), bovine RBC or other RBC. Other possible sources include recombinant enzymes or bioengineered enzymes.

Comparison of Different Approaches

Progress in therapy is a stepwise process and we now have more experience and knowledge in this area. We can now tailor-make blood substitutes ranging from simple oxygen carriers to complex nanobiotherapeutics (Table 19.1). However, if a condition only needs oxygen carrier then it would not be cost effective to use a more complex nanobiotherapeutic. On the other hand, it would be folly not to use a more complex nanobiotherapeutic if indicated. Table 19.1 is a brief summary comparing PolyHb, Poly-[Hb- SOD-CAT] and Poly-[Hb-SOD-CAT-CA] with RBC.

Potential Implications

Under normal circumstances, donor blood is the best replacement for blood. HOWEVER:

1. Natural epidemics (e.g., HIV, Ebola, Zika, COVID-19, etc.) or man-made epidemics (terrorism, war, etc.) can result in contaminated donor blood or disqualified disease contact donors. Unlike red blood cells (RBC) or the soluble form, lyophilized Poly-[Hb-SOD-CAT-CA] can be heat pasteurized in addition to ultrafiltration and glutaraldehyde crosslinking [13]. FDA has already approved the sterilization method used for PolyHb.
2. Severe blood loss from accidents, disasters, terrorism or war may require urgent blood transfusion that cannot wait for transportation to the hospital for blood group testing – especially in the frontline or remote area especially northern regions. Unlike RBC, Poly-[Hb-SOD-CAT-CA] and other HBOCs do not have blood groups and can be given on the spot.
3. In very severe hemorrhagic shock there is usually a safety window of 60 min for blood replacement, beyond which there could be problems related to irreversible shock. Our animal study shows that Poly-[Hb-SOD-CAT-CA] with enhanced RBC enzymes could prolong the safety window.
4. Heart attack and stroke can be caused by obstruction of arterial blood vessels. Unlike RBC particles, this nano-

Table 19.1 comparison of PolyHb, PolySFHb, Poly-[Hb-SOD-CAT], Poly-[Hb-SOD-CAT-CA] with RBC. From Chang [15] with copyright permission

Properties	Red blood cells (RBC)	Poly-[Hb-SOD-CAT-CA]	Poly-[Hb-SOD-CAT]	Poly-Hb	Poly-SFHb
Oxygen	7 microns RBC	Soluble oxygen carrier	Soluble oxygen carrier	Soluble oxygen carrier	Soluble oxygen carrier
Oxygen radicals	7 microns RBC Enzyme	3–6 times Enhanced antioxidant enzymes functions better removal of oxygen radicals	3–6 times Enhanced enzymes better removal of oxygen radicals	N/A	RBC enzymes no enhancement
Carbon dioxide	7 microns RBC Enzymes	3–6 times Enhanced carbonic anhydrase function Better CO_2 removal	N/A	N/A	RBC enzymes no enhancement
Storage stability	Suspension on <1-day 20C, 42 days 4C	lyophilized 320 days 20C, >320 days 4C	lyophilized 320 days 20C, >320 days 4C	Solution >2 years 20C	lyophilized 320 days 20C, >320 days 4C
Blood groups	Yes. unless O, Needs blood group matching and typing	None Can be given on the spot	None Can be given on the spot	None Can be given on the spot	None Can be given on the spot
Heat sterilization	Cannot	If lyophilized	If lyophilized	If lyophilized	If lyophilized
Availability	Limited human source	Material from human & nonhuman sources	Material from Human & nonhuman sources	Material from human & nonhuman sources	Material from human & nonhuman sources
Circulation time and areas of application	Donor blood: 30 days Better for elective long term RBC replacement But problem in severe sustained hemorrhagic shock and ischemic reperfusion	1–2 days for emergency uses & therapy in conditions requiring enhancement RBC functions as in severe sustained hemorrhagic shock and ischemic reperfusion	1–2 days for emergency uses & therapy in conditions requiring enhanced antioxidant functions as in ischemia reperfusion	1–2 days for emergency uses & therapy in conditions requiring only enhanced O2 transport	1–2 days for emergency uses & therapy but only same enzymes and have same problems as RBC in severe sustained hemorrhagic shock and ischemic reperfusion

RBC red blood cells,
Poly-[Hb-SOD-CAT-CA]: Polyhemoglobin-Superoxide dismutase-catalase-carbonic anhydrase Poly-[Hb-SOD-CAT]: Polyhemoglobin-Superoxide dismutase-catalase
Poly-Hb: Polyhemoglobin
Poly-SFHb: Poly-[stroma-free Hemoglobin or content of RBC with stroma removed]

biotherapeutic being a solution can more easily perfuse through partially obstructed vessels to reach the heart and brain to supply oxygen and remove tissue carbon dioxide. Furthermore, its enhanced antioxidant enzymes (SOD and CAT) can effectively remove oxygen radicals to prevent ischemia reperfusion injury to the reperfused tissues.

5. Red blood cells have to be stored in refrigeration thus more difficult to transport and store in disaster, frontline and remote areas. We show that Poly-[Hb-SOD-CAT-CA] solution can be stored at room temperature for more than 40 days, compared to RBC of 1 day at room temperature. The lyophilized form can be stored in refrigeration at 4C for more than 320 days as compared to RBC of 42 days.

Summary and Concluding Remarks

In clinical medicine, there is no one single approach for all conditions. Saline or colloid plasma expander may be enough for some conditions. When the clinical condition only requires an oxygen carrier like PolyHb, then there is no need to use the more complex and more expensive Poly-[Hb-SOD-CAT-CA]. Other conditions may only require oxygen carrier with antioxidant functions There are also conditions that may only require PolySFHb with the same enzymes functions as RBC. On the other hand, in severe sustained hemorrhagic shock, even red blood cells may not be enough to prevent irreversible shock and for this we may require Poly-[Hb-SOD-CAT-CA] with enhancement of all three red blood cell functions.

Translation to clinical use is very time consuming and we should learn from past experience not to wait until it is again too late. We should also analyze how research in animal studies can be applied to clinical use in patients. We also need to continue to look into the future since biological therapy undergoes rapid progress with many other future possibilities as discussed by many authors elsewhere [17–19].

Key Points

- A soluble nanobiotherapeutic, Poly-[hemoglobin-superoxide dismutase-catalase-carbonic anhydrase] with enhancement of all 3 red blood cell (RBC): transports oxygen, removes oxygen radicals and transports carbon dioxide. It is possible to have the enzyme concentration increased to 2, 4 and 6 times that of RBC.
- In a 90 min 2/3 blood volume loss hemorrhagic shock rat model, it is more effective than blood in the recovery of intracellular pCO2, cardiac ischemia, plasma lactate, troponin, histology of the heart and intestine.
- Four weekly 5% blood volume intravenous injections in rats followed by 30% exchange transfusion did not have any adverse effect on safety nor adverse immunological or anaphylactic response.
- The lyophilized form can be stored for 1 year at 4C (compared to 42 days for RBC) and 300 days at room temperature (compared to 1 day for RBC). Unlike RBC or the solution form, the lyophilized form can be heat pasteurized and retain enzyme activity.
- We have also developed a method to extract the needed enzymes from RBC at low costs.

Acknowledgement Material from this chapter comes from some of the text and figures of this author's papers [11, 13, 14, 16] published in Artificial Cells, Nanomedicine and Biotechnology (Taylor & Francis Publisher) with copy right permission.

The author's research in this area has been supported by the Canadian Institutes of Health Research and a partnership grant of the Canada Blood Service/Canadian Institutes of Health Research. Both are nonprofit organizations of the government of Canada. The opinions in this paper are those of the author and not necessary those of the granting agencies or the government of Canada.

This research is now being developed by a company with the author as consultant.

References

1. Chang TMS. Semipermeable microcapsules. Science. 1964;146:524–5.
2. Chang TMS. Stabilisation of enzymes by microencapsulation with a concentrated protein solution or by microencapsulation followed by cross-linking with glutaraldehyde. Biochem Biophys Res Commun. 1971;44:1531–6.
3. Keipert PE, Chang TMS. Pyridoxylated polyhemoglobin as a blood substitute for resuscitation of lethal hemorrhagic shock in conscious rats. Biomat Med Dev Artif Organs. 1985;13:1–15.
4. Chang TMS. Monograph on ARTIFICIAL CELLS: biotechnology, nanotechnology, blood substitutes, regenerative medicine, bioencapsulation, cell/stem cell therapy. World Scientific Publisher/Imperial College Press; 2007. 435 pages. http://www.medicine.mcgill.ca/artcell/2007%20ebook%20artcell%20web.pdf.
5. Mer M, Hodgson E, Lee W, Jacobson B, Levien L, Snyman J, Sussman MJ, James M, van Gelder A, Allgaier R, Jahr JS. Hemoglobin glutamer-250 (bovine) in South Africa: consensus usage guidelines from clinician experts who have treated patients. Transfusion. 2016;56(10):2631–2636009.
6. Moore E, Moore FA, Fabian TC, Bernard AC, Fulda GJ, Hoyt DB, et al. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. J Am Coll Surg. 2009;208:1–13.
7. D'Agnillo F, Chang TMS. PolyHb-superoxide dismutase-catalase as a blood substitute with antioxidant properties. Nat Biotechnol. 1998;16:667–71.
8. Ma L, Hsia CJ. Polynitroxylated Hb as a multifunctional therapeutic for critical care and transfusion medicine. In: Chang TMS, editor. Selected topics in nanomedicine. Singapore: World Science Publisher/Imperial College Press; 2013. p. 169–94.
9. Sims C, Seigne P, Menconi M, Monarca J, Barlow C, Pettit J, Puyana JC. Skeletal muscle acidosis correlates with the severity of blood volume loss during shock and resuscitation. J Trauma. 2001;51:1137–46.
10. Tronstad C, Pischke SE, Holhjem L, Tonnessen TI, Martinsen OG, Grimnes S. Early detection of cardiac ischemia using a conductometric PCO 2 sensor: real-time drift correction and parameterization. Physiol Meas. 2010;31:1241–55.
11. Bian Y, TMS C. A novel nanobiotherapeutical Poly-[hemoglobin-superoxide dismutase-catalase-carbonic anhydrase] with no cardiac toxicity for the resuscitation of a 90 minutes sustained severe hemorrhagic shock rat model with 2/3 blood volume loss Artificial Cells. Nanomed Biotechnol. 2015;43(1):1–9.
12. Guo C, Chang TMS. Long term safety and immunological effects of a nanobiotherapeutic, bovine poly-[hemoglobin-catalase-superoxide dismutase-carbonic anhydrase], after four weekly 5% blood volume top-loading followed by a challenge of 30% exchange transfusion. Artif Cells Nanomed Biotechnol. 2018;46(7):1349–63.
13. Bian Y, Guo C, Chang TMS. Temperature stability of Poly-[hemoglobin-superoxide dismutase–catalase- carbonic anhydrase] in the form of a solution or in the lyophilized form during storage at −80 °C, 4 °C, 25 °C and 37 °C or pasteurization at 70 °C. Artif Cells Nanomed Biotechnol. 2016;44:41–7.
14. Guo C, Gynn M, Chang. Extraction of superoxide dismutase, catalase and carbonic anhydrase from stroma-free red blood cell hemolysate for the preparation of the nanobiotechnological complex of polyhemoglobin-superoxide dismutase-catalase- carbonic anhydrase, journal artificial cells. Nanomed Biotechnol. 2015;43(3):157–62.
15. Chang TMS. Translational feasibility of soluble nanobiotherapeutics with enhanced red blood cell functions. Artif Cells Nanomed Biotechnol. 2017;45:671–6.
16. Wong N, Chang TMS. PolyHb-fibrinogen: a novel blood substitutes with platelet- like activity for extreme hemodilution. Artif Cells Blood Substit Biotechnol. 2007;35:481–9.
17. Chang TMS. ARTIFICIAL CELL evolves into nanomedcine, biotherapeutics, blood substitutes, drug delivery, enzyme/gene therapy, cancer therapy, cell/stem cell therapy, nanoparticles, liposomes, bioencapsulation, replicating synthetic cells, cell encapsulation/scaffold, biosorbent/immunosorbent hemoperfusion/plasmapheresis, regenerative medicine, encapsulated microbe, nanobiotechnology, nanotechnology. Artif Cells Nanomed Biotechnol. 2019;47(1):997–1013.
18. TMS C, Bülow L, Jahr J, Saika H, Yang CM, editors. Nanobiotherapeutic based blood substitutes. Singapore: World Science Publisher; 2021.
19. Related websites: www.artcell.mcgill.ca, www.artificialcell.info.

20

Paradigm Shift for Designing Oxygen Therapeutics: New Insights Emerging from Studies with Transgenic Mouse Models of Sickle Cell Disease

Synergy of Supra Plasma Expansion and High O_2 Affinity of Blood Substitutes

Seetharama Acharya, Craig Branch, Amy G. Tsai, and Marcos Intaglietta

Abbreviations

BOLD	Blood oxygen level dependent
CBF	Cerebral blood flow
EAF	Extension Arm Facilitated
FCD	Functional Capillary Density
Hb	Human hemoglobin
MAP	Mean arterial pressure
MP	Maleimide PEG modified Hb
MP4	Maleimide PEG modified Hb constituted as a 4 gm % solution,
MP8	Maleimide PEG modified Hb constituted as 8 gm % solution
MRI	Magnetic resonance imaging
PEG polyethylene glycol, PEGylation-conjugation of PEG-chains P5K2, P10K2, P5K4, P3K6, P5K6	The PEG-Hb conjugates studied here has been defined by the general formula PxKy, where 'P' represents PEG chain of mass 'x'in kilo daltons, and K represents number of copies represented by 'y' conjugated to a given Hb molecule
RBC	Red blood Cells

S. Acharya (✉) · C. Branch
Departments of Medicine, Physiology and Biophysics, and of Radiology Albert Einstein College of Medicine, Bronx, NY, USA

Department of Bioengineering, UCSD, San Diego, CA, USA
e-mail: seetharama.acharya@einsteinmed.org

A. G. Tsai · M. Intaglietta
Department of Bioengineering, UCSD, San Diego, CA, USA

Introduction

The RBC serves a dual role in oxygen delivery to tissues, it carries oxygen from lungs to tissues and modulates the blood flow/microcirculation through blood shear thinning to achieve a proper delivery of oxygen [1]. However, design of Hb derivatives as oxygen therapeutics with an oxygen affinity comparable to or lower than that of Hb in RBC has focused on increasing the oxygen carrying capacity of blood to compensate for blood loss or anemia. Multiple intrinsic properties of blood and Hb inside the RBC modulate the hyper/hypotensive activity of circulatory system to optimize oxygen delivery. In designing our PEG-Hbs as non-hypertensive solutions we departed from the original paradigm. We have combined the potential advantages of high oxygen affinity of Hb for targeted oxygen delivery to oxygen starved tissues with intrinsic improvements in perfusion properties induced by colloidal plasma expanders that afford in situations of anemia in designing our new oxygen therapeutics to mitigate the vasoconstriction mediated acellular Hb. This has necessitated the use of lower amounts of oxygen therapeutics, and this represents a complete paradigm shift.

In this chapter we focus the attempt to mimic the dual role of RBC in circulation with the designed molecules. Here, we review the development of new strategies to endow these dual RBC-like properties to the new oxygen therapeutics. This new class of oxygen therapeutics represents novel biomaterials, nano-oxygen pumps, that increase the efficacy of oxygenation of Hb (in flow) in RBCs in the lungs and oxygen delivery (out-flow) from RBCs in the tissues when the level of Hb (hematocrit) in the blood drops to the transfusion trigger levels or below. The new platform for treating anemia recommends the use of lower amounts (dosage) of these new semisynthetic biomaterials as opposed to the use of larger dose (compensate for blood loss or anemia) as strategized

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_20

with conventional low oxygen affinity oxygen therapeutics. This paradigm shift is needed since the new high oxygen affinity biomaterials increase not only increase tissue oxygenation through better extraction of oxygen from the RBC in the tissues, and in doing so release NO, a vasodilator. As a consequence, increase in tissue oxygenation by these nano-oxygen pumps resembles the normal oxygenation through RBC with normal hematocrit levels but through improved oxygen extraction rather than as envisioned by blood substitutes. Accordingly, these nano oxygen pumps have a self-activated mechanism for improving perfusion/microcirculation coupled with improved oxygen extraction. Besides, they also increase the oxygen saturation in the lungs, thus becoming a mechanism for improving the oxygen carrying capacity of Hb within RBC as it gets attenuated by anemia, an approach very distinct from the old paradigm advocating increased the oxygen carrying capacity of circulatory system (to restore the lost oxygen carrying capacity either completely or partly) to increase oxygen delivery.

The delineation of these new concepts has been facilitated by the development of new PEGylated Hbs. A new platform for PEGylation of proteins has been developed at Einstein. referred to as extension arm facilitated (EAF) PEGylation (EAF PEGylation). This platform is essentially a protein thiolation facilitated PEGylation protocol [2, 3]. The first product of this platform is EAF P5K6 Hb with six copies (K) of PEG, denoted by letter P, of mass 5000. Sangart version of hexa-PEGylated Hb is referred as maleimide PEG Hb, (MP) with the tradename Hemospan (see below for full description of the platform). A 4 gm % solution of Hemospan is referred to as MP4, and an 8 gm % solution as MP8, but MP4 is the frequently used formulation. It may be noted the concentration of Hb in MP4 is at least threefold lower than in previously designed oxygen therapeutics. In EAF P5K6 Hb we have two PEG-5Kchains directly conjugated to Cys-93(β) and rest are conjugated on the extension arms built on ε-amino groups of Hb. We have a di-PEGylated Hb with two copies of PEG 5 K on Cys-93(β) conjugated directly, P5K2 Hb. P10K2 Hb is a version di-PEGylated Hb generated using maleimide PEG-10,000 instead of maleimide PEG-5000. EAF P3K6 Hb is a newer version of hexa-PEGylated Hb generated using maleimide PEG-3000 using extension arm chemistry, to have a smaller PEG-shell. These aspects are discussed in detail in the Chapter.

Development of EAF P5K6 Hb and Its Prototype Hemospan as an Oxygen Carrying Colloidal Plasma Expanders

In designing the PEG Hb as a new class of oxygen therapeutics, we at Einstein had the following structural and functional properties for Hb derivatives as guiding principles for our development. This has led to the design of Extension Arm Facilitated (EAF) PEGylation platform conjugating PEG-chains to proteins (Fig. 20.1).

(i) Design of a new PEGylation strategy to surface decorate Hb such that it will induce very limited perturbation of the hydration layer of Hb and does not weaken the inter-dimeric interactions of the tetramer. Besides, it should neither perturb the overall surface charges of the molecule nor weaken the intra or interdimeric interactions of Hb. Hexa-PEGylation of Hb using six copies of PEG 5K chains by a novel Extension Arm Facilitated (EAF) PEGylation, EAF P5K6 Hb platform appears to accomplish all these structural aspects [2, 3].

(ii) The PEGylated molecule exhibits a hydrodynamic volume comparable to a polymeric form of Hb with three to four copies of the tetrameric Hb unit and thus avoids extravasation in the circulation. Though the total mass of PEG conjugated Hb is only 30 K, the Hb surface decorated with six copies of PEG 5K chains by EAF PEGylation (EAF P5K6 Hb) exhibits a molecular radius of ~ 6.5 nm. The elution pattern of EAF P5K6 Hb on molecular sieve chromatography is sharper than polymerized bovine Hb even though the average hydrodynamic volume of the two is comparable.

(iii) Lowering of hematocrit due to blood loss has an intrinsic vaso-constrictive impact because of reduced shear thinning of RBC; however, this hematocrit reduction induced vaso-constriction can be partially compensated or attenuated by conventional colloidal plasma expanders with viscosities around 2.8 cp and compensated even better with colloidal plasma expanders with viscosities around that of blood (5 cp) or higher than 5.0 (Supra Plasma Expanders).

(iv) Previously designed oxygen therapeutics are low-oxygen affinity Hbs with affinities comparable to that of the RBC or lower. These will release their oxygen early at the arteriolar level causing the vessel to vaso-constrict thereby limiting microcirculatory perfusion. In contrast, PEG Hbs are generally high oxygen affinity Hbs and thus were chosen as they will be applied to target oxygen delivery to hypoxic regions in the body. PEGylation of proteins is viscogenic and thus can compensate for the vasoconstrictive activity of Hb to some degree. PEGylated Hb and its prototype PEG Alb are novel semisynthetic hybrid biopolymers with ordered and disordered regions and with novel molecular properties yielding non-hypertensive colloidal plasma expanders. These represent a novel class of oxygen therapeutics that mimic some of the properties of erythrocytes.

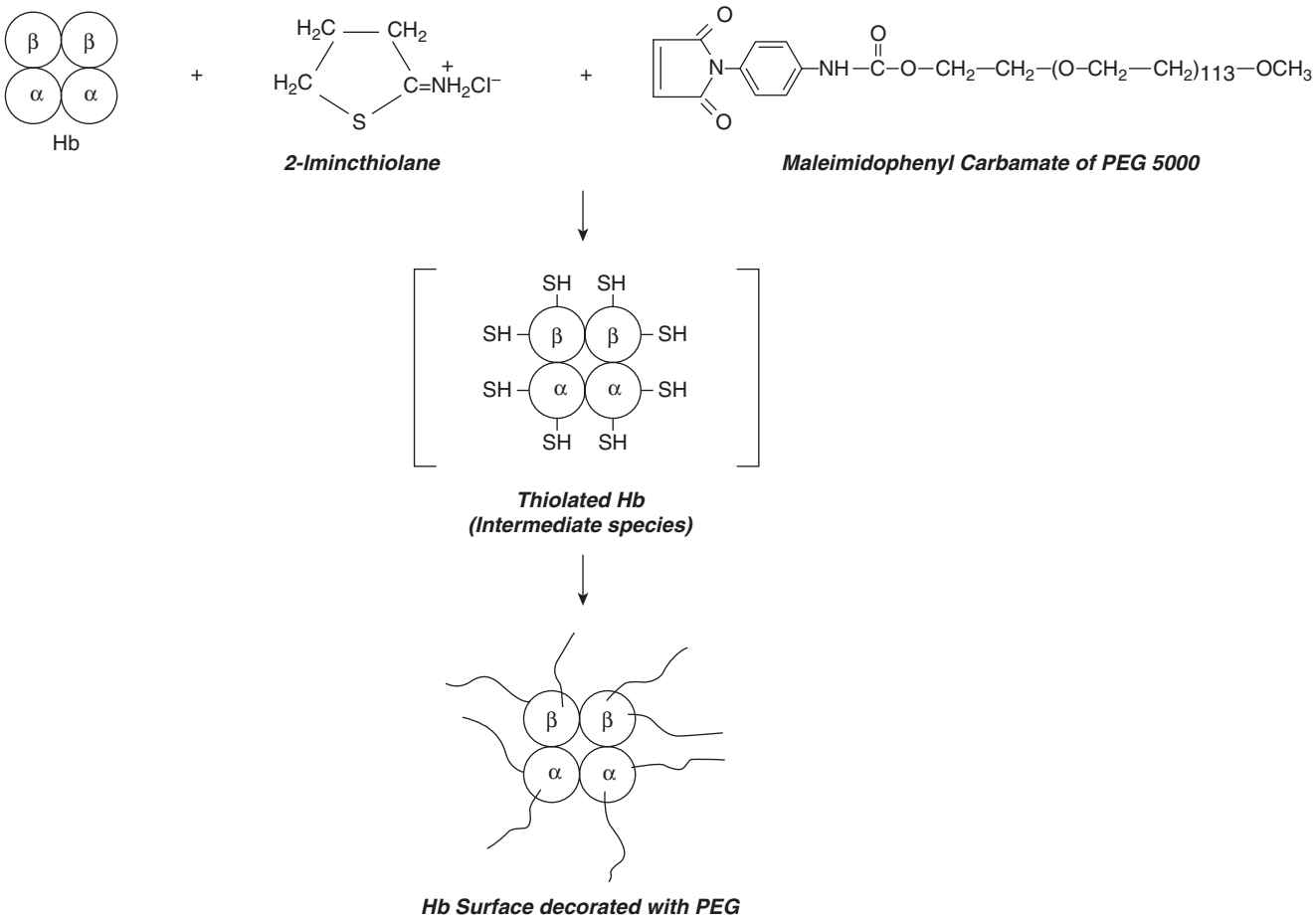


Fig. 20.1 Schematic representation of Extension Arm Chemistry Facilitated PEGylation

Interdimeric Interactions of Hb PEGylated Using Extension Arm Chemistry

Earlier versions oxygen therapeutics are essentially intramolecularly crosslinked Hbs with the crosslinking between like chains, i.e., between αβ dimers of the tetramers or polymeric Hbs to avoid nephrotoxicity. Direct PEGylation of un-crosslinked Hb induces a weakening of interdimeric interactions of tetrameric Hb as seen with Enzon PEG-Hb, a PEGylated bovine Hb with ten copies of PEG-5K chains. However, very little nephrotoxicity is seen with Enzon PEG-Hb, showing PEGylated αβ-dimers are not readily filtered through kidneys. We have compared the influence of PEGylation on hexaPEGylated Hb (PEGylated with PEG-5K) on the inter-dimeric interactions of human Hb. HexaPEGylation of Hb with PEG-5K weakens the interdimeric interactions in a PEGylation chemistry dependent fashion [4–6]. The hydrodynamic volume of PEGylated αβ-dimers with an average of three PEG-5K chains (molecular mass of αβ-dimers is 48K and lower than tetrameric Hb) present in a sample of hexaPEGylated Hb is significantly higher than intramolecularly crosslinked Hb, i.e., PEG-chains conjugated onto Hb are essentially disordered. Accordingly, there is very little nephrotoxicity with PEGylated Hbs with six or more PEG 5K chain. Concomitant with this weakening of inter-dimeric interaction on PEGylation, direct PEGylation increases the oxygen affinity and abolishes cooperativity.

In EAF PEGylation described above, two of the six PEG-chains are directly conjugated to Hb through the side chain functions, thiol group of Cys-93(β) and remaining four PEG-chains are conjugated on the thiol group of the extensions introduced on the surface amino groups of Hb [7]. We can protect reversibly thiol group of the Cys-93(β) as mixed disulfide during EAF PEGylation of Hb and this will generate EAF PEG-Hb wherein all PEG-chains are conjugated only through thiol group on the extension arm [5, 6] or by carrying out the reaction in deoxy conformation [2, 7].

On the contrary, in EAF P5K6 Hb, the strength of interdimeric interactions of Hb remains essentially unperturbed (Fig. 20.2). The absence of PEGylation on Cys-93(β) does

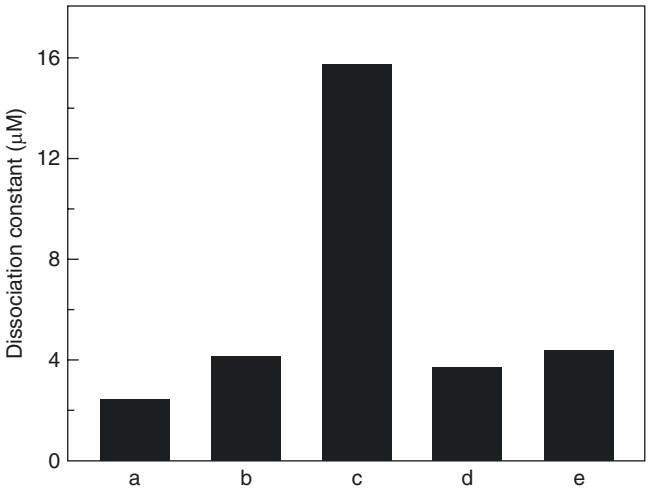


Fig. 20.2 The tetramer-dimer dissociation constant of the EAF-PEG-Hb. HbA (*a*), the PEG-Hb with free Cys-93(β) (*b*), the PEG-Hb without free Cys-93(β) (*c*), Hb- NEM modified in the presence of iminothiolane with free Cys-93(β) (*d*), and Hb NEM modified in the presence of iminothiolane without free Cys-93(β) (*e*) were loaded on the Superose 12 column for the dissociation constant measurement

not increase the stability of PEGylated Hb to any noticeable level. The factors influencing the choice of EAF P5K6 Hb as the best molecule for our studies are: (i) Overall stability of the molecule (ii) High oxygen affinity (iii) significant level of cooperativity, and (iv) Absence of the Bohr effect. Many of these molecular and functional properties of EAF P5K6 Hb are counter intuitive to the desired properties for a blood substitute as per old paradigm for the design of oxygen therapeutics.

The potential advantages of PEGylation induced plasma expander like properties of PEG Hb and the high oxygen affinity in an oxygen therapeutic has been discussed in detail by Winslow [8]. Based on observed viscosity of solutions of EAF P5K6 Hb as a function of protein concentration, it has been constituted as a 4 gm % solution and at this concentration, a solution of EAF P5K6 Hb has a viscosity comparable to that of conventional colloidal plasma expanders. At 4 gm %, EAF P5K6 Hb has a viscosity around 2 cp, slightly lower than the viscosity of conventional colloidal plasma expanders.

It may be noted here that a 4 gm % solution of EAF P5K6 Hb represents significantly a lower concentration of Hb as compared to the previous blood substitutes designed as per old paradigms and which are constituted as 12 gm % (or more) solutions. Given the high oxygen affinity of this PEG Hb and the lower concentration of EAF P5K6 Hb in the designed product solution, there has been significant skepticism in the scientific community in the ability of PEG-Hbs to deliver oxygen.

Hemospan (MP4) Is Non-toxic in Phase III Clinical Trial, However Not Superior to Conventional Colloidal Plasma Expander

Sangart licensed the platform for EAF hexa-PEGylation of Hb from Einstein and developed its blood substitute candidate under the trade name Hemospan [9].

However, Sangart introduced two significant changes into the platform licensed from Einstein, in preparing their molecule, Hemospan. First, instead of using chromatographically purified Hb, Sangart used stroma free Hb prepared from outdated blood from blood banks. Second, Sangart used a two-step procedure for EAF PEGylation instead of the one step platform considered to be better. Stroma free Hb was first reacted with iminothiolane at a protein concentration of 1 mM (in the one step platform practiced at Einstein Hb is reacted at 0.5 mM Hb concentration) and then mixed with PEG-5K maleimide. The major limitations of the two-step approach are: (i) a higher level of thiolation of Hb due to the higher protein concentration, and (ii) the thiolation reaction is done under oxy conditions in the absence of PEG maleimide. Accordingly some degree of air oxidation of thiol groups generated on Hb is expected in the two-step PEGylation process and also to generate intra and/or intra molecular crosslinks before the PEG maleimide is added to the reaction mixture as the second step of EAF PEGylation. In the one step platform, thiolation is done in the presence of PEG maleimide and thiol groups react with PEG maleimide as they are generated *in situ*.

Hemospan generated from stroma free Hb carried nearly 8 copies of PEG 5K chains compared to six PEG-5K chains in EAF P5K6 Hb [9] and exhibited molecular radius around 10 nm. Hemospan exhibits a higher oxygen affinity as compared to EAF P5K6 Hb and does not exhibit any cooperativity. The viscosity at 4 gm/dl (MP4) is around 2.2 cp and noticeably higher compared to 4 gm % EAF P5K6 Hb. At 8 gm/dl (MP8) of hemospan, the viscosity is around 5.2 cp and comparable to that of blood. The molecular radius of Hemospan was calculated from the colloidal osmotic pressure of the solution which is a colligative property. The molecular radius value for EAF P5K6 Hb is calculated from dynamic light scattering, a molecular property. Given the fact that interaction of EAF P5K6 Hb is comparable to native Hb, the higher molecular radius calculated for Hemospan suggests the presence of some PEGylated dimers due to the two-step preparation of EAF PEGylated Hb.

On the positive side, Hemospan was essentially free of Hb dependent toxicity in clinical trials. In phase III clinical trial with orthopedic surgery patients, Hemospan behaved essentially as colloidal plasma expanders in terms of mortality; subsequently Sangart did not pursue regulatory approval as a drug in view of the prohibitive cost of Hemospan as a colloi-

dal plasma expander. However, the clinical trial data indicated that Hemospan was superior to colloidal plasma expander hetastarch by showing (i) A lower number of hypotensive episodes (ii) A shorter length of hypotensive episodes. The data suggest that hemospan provides a better protection presumably either through targeted oxygen delivery to hypoxic areas, or through supra plasma expander activity of MP4 (see below), the primary objective of the designed molecule.

Accordingly, Sangart was moving forward with the clinical trial albeit with a different end point; reduction in ischemia mediated effects by following changes in lactic acid production. Though phase II trials provided positive results, Sangart could not raise the funds to pursue phase III clinical trial, returned the patents to Einstein and closed its operation. Therefore, a remaining unanswered question is whether a 4 gm % solution MP4 (a low level of Hb or very low level of high oxygen carrying capacity) with its high oxygen affinity Hb has contributed to the absence of therapeutic benefits of Hemospan over hetastarch in the Phase III clinical trials or the design of EAF P5K6 Hb is tuned properly to achieve improved oxygen delivery.

EAF P5K6 Hb Is a Semisynthetic, Colloidal Supra Plasma Expander

As noted above vaso-constrictive effect induced by a decrease in hematocrit level (or anemia) could be off-set to some level by conventional colloidal plasma expanders like dextran 70. Surprisingly, when EAF P5K6 Hb (or MP4) tested in hamster extreme hemodilution models (hematocrit levels 11%; severe anemia), the functional capillary density and blood flow seen is noticeably better than seen with conventional colloidal plasma expanders like dextran-70 at 6 gm % (2.8 cp) (Table 20.1). On the other hand, it is not as good as with the supra plasma expander dextran 500 at 6 gm % (5.2 cp). The increased functional capillary density and high blood flow arising from the vaso-dilatory influence of supra plasma expanders. This vaso-dilatory influence seen with high density colloidal plasma expander has been attributed to the increased shear thinning of blood and the resultant increased intrinsic NO production by the endothelium. Given the fact the viscosity of a 4 gm % MP4 (or EAF P5K6 Hb) is lower than dextran 70, it is clear that the supra plasma expansion activity of EAF P5K6 Hb is, apparently, a consequence of some unique structural aspect of this semisynthetic conjugated protein. A schematic representation of this semisynthetic supra plasma expander is shown in the Fig. 20.3. The packing density of central protein core, which is a compactly folded protein, is higher than the PEG-shell engineered outside and the PEG shell is placed outside the hydration layer of protein through a zone of extension arms. The essentially disordered PEG chains give a degree of flexibility (ability to change shape) as a function of hydrostatic pressure, i.e., these semisynthetic molecules are endowed with a degree of

Table 20.1 Influence of pattern of PEGylation of Hb on microcirculation parameters

Sample	FCD	Flow (normalized to baseline)	Tissue PO2 (mm Hg)
Dextran 70	0.38	0.80	1.1
Dextran 500	0.71	1.20	1.6
P5K2 Hb	0.77	0.93	7.0
P10K2 Hb	0.79	0.95	6.4
P5K4 canine Hb	0.71	0.79	2.5
P5K4 rHb (αH20C)	0.62	0.69	6.1
MP4	0.68	0.62	1.8
MP8	0.71	0.55	4.1

FCD functional capillary density, *rHb* recombinant Hb, *αH20C* histidine at position 20 in α-globin mutated to Cysteine. MP4 EAF P5K6 Hb of Sangart constituted as 4 gm % solution, MP8 EAF P5K6 Hb of Sangart constituted as 8 gm % solution

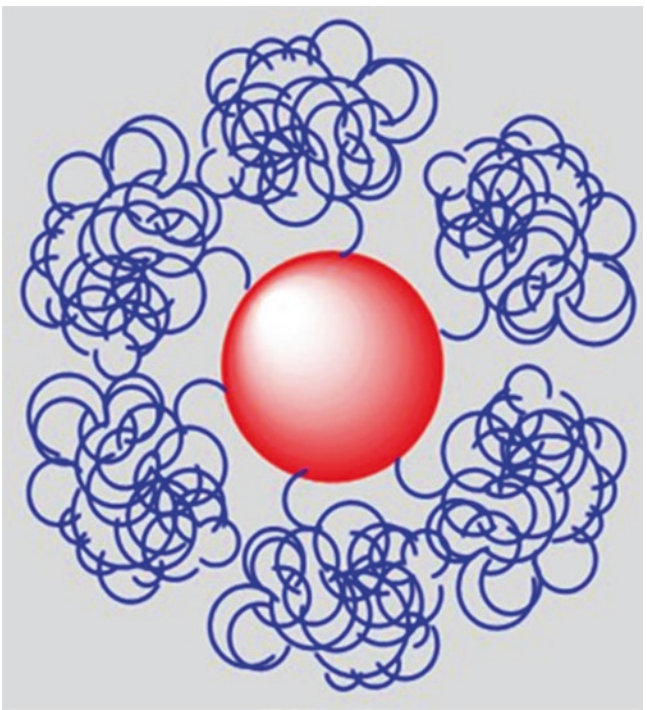


Fig. 20.3 Schematic representation of the six PEG-5K PEG-shell engineered onto Hb or Alb. The central core represents the protein (either Hb or Alb) where the polypeptide chains are packed compactly, ie., the atoms are packed densely. In the outer PEG-shell the six PEG chains are more disordered compared to central protein core. The outer PEG-shell contributes a mass of only 30 K of the total mass of 94 K, even though they occupy more space as compared to the protein core contributing to a mass of 64 K. Accordingly, the packing density of atom in PEG-shell is lower compared to that in protein core. The differential packing densities of the two regions of the molecule gives a degree of pseudoplasticity to these PEGylated molecules when they are in blood flow

pseudoplasticity. It may be noted that pseudoplasticity is an intrinsic property of RBC. Accordingly, these semisynthetic nanomaterials placed in plasma are "mini-RBCs", i.e., oxygen carrying nanoparticles (with a molecular radius around 6–7 nM). The pseudoplasticity and the high oxygen affinity of EAF P5K6 Hb distinguishes it from polymeric Hbs designed as oxygen therapeutics even though seems to have nearly comparable hydrodynamic volume.

Tissue Oxygenation with Supra Plasma Expanders in Situations of Low Hematocrit

In extreme hemodilution, the supra plasma expansion seen with dextran-500 is not accompanied by a noticeable increase in the level of tissues oxygenation. The inability of dextan-500 to improve tissue oxygenation reflects the level of anemia, i.e., overall poor (low) oxygen carrying capacity of the system (the hematocrit 11%, 4 gm % Hb), is too low to establish an oxygen gradient to facilitate the diffusion of oxygen from RBC through plasma to tissue even with the higher surface area and diminished blood flow.

The extreme hemodilution in hamsters essentially represents an experimentally induced severe anemia except for the fact the circulatory system has been partly compensated/stabilized by replacing the lost volume of blood with a conventional colloidal plasma expander with a viscosity around 2.8 cp. This model has served as a good system for mapping the oxygen therapeutics induced attenuation/improvement in mean arterial pressure, microcirculation, and improvement in tissue oxygenation, because the system is near to the critical oxygen carrying capacity.

MP4 (Hemospan at 4 gm %) is a supra plasma expander, superior to conventional colloidal plasma expanders (dextran-70). This is reflected by improved functional capillary density and microcirculatory blood flow. However, the tissue oxygenation seen with it is only marginally better than that seen with dextran 70 (Table 20.1). On the other hand, the microcirculatory response of MP8, (8 gm % solution of Hemospan) and with a viscosity comparable to dextran-500, did noticeably improve tissue oxygenation. It may be noted that the concentration of Hb in plasma with MP8 is only around 45% higher than that with MP4, not double. The colloidal osmotic pressure of MP8 is very high, and therefore a significant auto transfusion is induced by this molecule. This reflects the intrinsic potential of the high oxygen affinity EAF P5K6 Hb to deliver oxygen in situations of severe anemia.

EAF Hexa-PEGylation Induced Supra Plasma Expansion Activity of Alb

EAF P5K6 Alb has been generated as a prototype of EAF P5K6 Hb to map whether EAF hexa-PEGylation with maleimide-phenyl PEG-5K can induce supra plasma expansion. It may be noted that Alb and Hb have comparable molecular size, and both are globular proteins. Indeed, a 4 gm % solution of EAF P5K6 Alb gives a microcirculatory response comparable to MP4, but a flow rate higher than Hemospan. A 4 gm % just as EAF P5K6 Hb. Interestingly, it induces an increase in endothelial NO production in much the same way as high viscosity dextran-500 [10]. This is accomplished by increasing the shear thinning of blood. EAF hexa-PEGylation of Hb and Alb induces supra plasma expansion activity to these proteins. Engineering supra plasma expansion activity to Hb by EAF hexa-PEGylation coupled with the high oxygen affinity represents a major paradigm shift in the design of oxygen therapeutics discussed in this Chapter.

Hypertensive Activity and Tissue Oxygenation by Hb Modified at Cys-93(β) with Maleimide PEG and with High Oxygen Affinity

Intramolecularly crosslinked Hb, Bis succinimidyl PEG 10 K Hb through Cys-93(β) where the spacer arm of the crosslinker (PEG-chain) is located outside the central cavity of Hb [2] may be considered as an isomeric form of di-PEGylated Hb, PEGylated at its Cys-93(β) (P5K2 Hb). Both exhibit comparable high oxygen affinity Hb. Interestingly, the hydrodynamic volume of Bis succinimidyl-PEG 10,000 αα-fumaryl Hb as reflected by size exclusion chromatography is smaller than that of Bis succinimidyl PEG-10,000 Hb, and the latter is smaller than P5K2 Hb [11]. Two copies PEG-5K chains on Hb with its free distal end are more disordered than the spacer arm than in the intramolecularly crosslinked Hb, Bis Mal PEG-10 K.

The P5K2 Hb is non-hypertensive at 4 gm % and even though it has a viscosity of only 1.4 cp [11], and it maintains an excellent functional capillary density and blood flow in extreme hemodilution studies in the experimentally induced severe anemia hamster model [12]. When used at 4 gm/dl, P5K2 Hb with a viscosity just around 1.4 cp the functional capillary density and blood flow are in fact far better than dextran 70 control (with viscosity around 2.8 cp), and it may be noted that the high oxygen affinity Hb improves tissue oxygenation even though the amount of PEG Hb in plasma is only about 1 gm, and only about 20% of the total Hb in circulation (which is at nearly ~5 gm/dl). Nearly 4 gm/dl of the total Hb is coming from the RBC. Thus, a high oxygen affinity PEG Hb improves tissue oxygenation under conditions of severe anemia even though the level of the high oxygen affinity PEG Hb in plasma is only 20% of the total Hb in circulation. This represents about 10% of the total blood loss (about 8–10 gm) in hamster in extreme hemodilution studies that has been replaced as P5K2 Hb in the plasma. The microcirculation when P5K2 Hb is present in plasma, as represented by functional capillary density and blood flow, is also

better than with dextran 70 control. P10K2 Hb with a PEG mass conjugated to Hb double than that in P5K2 Hb also gives essentially identical results. When we can significantly improve the tissue oxygenation, the increase in the viscosity of the test solution of PEG Hb by itself contributes very little to improvement in microcirculation making dominant role of improved tissue oxygenation in dictating the microcirculation/perfusion readily apparent.

Pattern of PEGylation on PEG Hb Dictates the Efficiency of Tissue Oxygenation During Extreme Hemodilution

Direct PEGylation of Hb with maleimide on the reactive free thiols of Cys-93(β) in Hb (P5K2 Hb, P10K2 Hb or P20K2) was found to attenuate the intrinsic vasoconstrictive activity of Hb [11] as an inverse function of the PEG mass conjugated in top-load experiments with hamster. EAF P5K6 PEGylation of Hb provided the best non-hypertensive activity while P20K2 Hb provided the least attenuation of vasoconstrictive *activity*. Nonetheless, Hb with two PEG-5K chains, exhibited noticeable attenuation of the hypertensive activity of Hb, in comparison to control unmodified Hb [11]. Even though the oxygen affinity difference between EAF P5K6 Hb and P5K2 Hb is only marginal, the differences in tissue oxygenation level between P5K2 Hb and EAF P5K6 Hb is significant. Surprisingly, in extreme hemodilution studies we found that the functional capillary density and blood flow (i.e., microcirculation) does not correlate with the level of PEGylation or the oxygen affinity. We concluded that tissue oxygenation by the high oxygen affinity PEGylated Hb is impacted inversely by the amount of PEG in the PEG-shell of Hb, even though the overall effect of PEGylation on oxygen affinity appears to be minimal.

PEG-Shell of High Oxygen Affinity of EAF P5K6 Hb Attenuates the Efficiency of Tissue Oxygenation

In extreme hemodilution, tissue oxygenation obtained by P5K2 Hb is better in comparison to supra plasma expander, dextran 500 (Table 20.1). Though functional capillary density with these two preparations is comparable, the blood flow is noticeably better with dextran 500. When severe anemia is induced in hamster (4 gmHb/dl), a significant reduction in the oxygen carrying capacity (about 66 %) of the circulatory system, supra plasma expansion by itself is unable to significantly improve the oxygen delivery to tissues. Increasing the oxygen carrying capacity of the circulation in this extreme hemodilution model with 1 gm/dl of P5K2 Hb in plasma (20% the total overall oxygen carrying capacity) improves the tissue oxygenation, while the same amount of MP4 placed in plasma does not provide this therapeutic benefit. On the other hand, comparing MP8 to dextran 500 (with comparable viscosities), the MP8 is nearly as good at improving functional capillary density as MP4, but blood flow is noticeably lower compared to dextran 500. But MP8 improves tissue oxygenation better than dextran 500, although not as effectively as P5K2 Hb. Thus, at least in situations of severe anemia, the tissue oxygenation by high oxygen affinity PEG Hbs is not just a function of oxygen carrying capacity placed in plasma.

The increased oxygen affinity of P5K2 Hb is essentially a function of maleimide modification of Cys-93(β) while the contribution of the PEG-chains on the maleimide moiety in increasing the oxygen affinity appears to be marginal, if any. On the other hand, as multiple copies of the PEG-chains are conjugated to Hb and larger PEG-shell is engineered around Hb, further small increase in the oxygen affinity can be seen. But the influences of the maleimide modification of Cys-93(β) and the number of conjugated PEG-shells upon oxygen affinity do not seem to be additive or synergistic. The intrinsic oxygen affinity changes of PEG-Hbs is less than additive compared to the influence of maleimide modification of Cys-93(β) and of PEG-shell. Besides, as the number of copies of PEG-chains in the PEG-shell increase, the influence of allosteric effectors which to reduce the intrinsic oxygen affinity of Hb is significantly attenuated: increase in the destabilization (access to deoxy conformation) of oxy conformation by PEG shell occurs as the PEGylation increases. This is reflected as decrease in the delivery of oxygen. Tissue oxygenation by high oxygen affinity Hbs is not a direct correlate of the oxygen affinity.

Previous geminate recombination studies have shown that both maleimide modification of Cys-93(β) of Hb [13, 14] and the PEG-shell independently stabilize the oxy conformation and destabilize the deoxy conformation. Destabilization of the deoxy conformation is reflected by the observation that as the PEGylation increases allosteric effectors mediated lowering of the oxygen affinity of PEG Hb is [4] attenuated. Once Cys-93(β) of Hb is modified by a maleimide, the allosteric modification of oxygen affinity of Hb via modulation of geminate recombination by inositol hexa-phosphate (IHP) is essentially absent as compared to unmodified Hb.

These molecular aspects of reversible oxygen binding to PEG-Hbs could explain the lower efficacy of MP4 to improve tissue oxygenation in extreme hemodilution studies as compared to P5K2 Hb or P10K2 Hb. As noted earlier, the changes introduced by Sangart in the EAF PEGylation platform could have also contributed further to its lower efficacy. EAF P5K6 Hb prepared at Einstein provided a better tissue oxygenation as compared to MP4, however, its efficacy is noticeably lower than that of P5K2 Hb. The molecular dimensions of P5K2 Hb and EAF P5K6 Hb are significantly different, and we reason that the larger PEG-shell contributes to attenuation of the efficiency of oxygen release to tissues by PEG Hb, in spite of their contribution to induce supra plasma expansion activity to Hb.

High oxygen affinity of Hb is essentially a property of oxygen storage proteins like myoglobin. Increasing the oxygen affinity of Hb will have no influence on the oxygen carrying capacity of Hb since the oxygen tension at the lungs is very high due to high oxygen concentration in inspired air. While oxygen bound to Hb is unaltered, we anticipate that in the region where high oxygen affinity PEG-Hbs have to release their oxygen, tissue oxygenation will be a function of the oxygen affinity of the oxygen therapeutic placed in plasma and in particular stability of the deoxy conformation of PEG-Hbs, as well as the local tissue oxygen tension. The concentration of oxygen therapeutic in plasma represents the increased oxygen carrying capacity introduced to off-set lowered oxygen carrying capacity due to reduced hematocrit, in extreme hemodilution 11% instead of normal around 45%. The concentration of oxygen therapeutic in plasma should be high enough to build sufficient oxygen tension in the plasma to facilitate the diffusion mediated release of oxygen from plasma to tissues. Consideration of the structure of PEG-shell design, particularly the influence on- and off rates of oxygen from Hb is thus very important. Therefore, the molecular structure of engineered PEG-shell of PEG-Hb can contribute to attenuation of oxygen delivery by reducing the stability of deoxy conformation. This is apparent as evidenced by MP4 and the P4K2 design and the oxygen delivery by thesemolecules in extreme hemodilution. Even though MP4 did not improve tissue oxygenation, MP8 did improve the tissue oxygenation even though it is not as efficient as P5K2 Hb (at 4 gm %). The COP of MP8 is very high and this results in a significant auto transfusion and increased plasma volume, thereby the concentration of PEG Hb in plasma is not a direct correlate of Hb concentration in the test solution. The structural aspects of PEG-Hb coupled with increased plasma volume lowers the overall efficacy of tissue oxygenation when high oxygen affinity PEG Hb is used as oxygen therapeutics.

Design of EAF P3K6 Hb and Its Ability to Improve Tissue Oxygenation

One of the important differences between P5K2 Hb and EAF P5K6 Hb is the difference in molecular dimensions (hydrodynamic volume). Accordingly, we decided to evaluate the influence of the hydrodynamic volume of the PEG-shell on Hb generated by surface decoration of Hb with multiple copies of PEG-chains to improve tissue oxygenation. We decided to start with hexa-PEGylation to keep the number of PEG-chains conjugated to Hb same, six and reduce the molecular mass of PEG-chains to 3000 and generated EAF P3K6 Hb. This involves simply replacing maleimide PEG-5K with maleimide PEG-3K in Einstein EAF PEGylation platform.

Molecular and solution properties of EAF P3K6 Hb is compared with that of EAF P5K6 Hb in Table 20.2. The functional properties of EAF P3K6 Hb are essentially comparable to EAF P5K6 Hb except for the molecular and solution properties are distinct. This is anticipated because of the lower mass of PEG conjugated to Hb which is only 60% the mass of conjugated PEG in EAF P5K6 Hb. Compared to PEG-5K chains, the PEG-3K chains pack (organize/interact) much more compactly on the surface of Hb in the PEG-shell of EAF P3K6 Hb. The molecular shape of EAF P3K6 Hb as defined by molecular modelling appears to be more globular while EAF P5K6 Hb is more ellipsoidal.

Table 20.2 Molecular and functional properties of EAF hexa-PEGylated Hbs

Molecules	Radius (nm)	Molecular volume $(nm)^3$	Packing density of PEG shell	Oxygen affinity P50 mm Hg
HbA	3.0	116.6	–	14
EAF P3K6 Hb	4.9	493.0	47.7	8
EAF P5K6 Hb	6.5	1150.0	29.0	7–8

In view of the lower viscosity of 4 gm % solution of EAF P3K6 Hb compared to EAF P5K6 Hb, we used 6 gm % solution of EAF P3K6 Hb to evaluate the influence of PEG shell size on the tissue oxygenation in the extreme hemodilution in hamster. EAF P3K6 Hb increases the tissue oxygenation slightly better than that seen with P5K2 Hb or P10K2 Hb. This clearly reflects the influence of PEGylation pattern on the efficacy of PEG Hb to deliver oxygen. Even though the COP of a 6 gm % solution of EAF P3K6 Hb is higher than EAF P5K6 Hb and MP4, the concentration of EAF P3K6 Hb in plasma is just about 50% higher than that with MP4 or P5K2 Hb: increase in auto-infusion seems to be limited with EAF P3K6 Hb as this this increase plasma EAF P3K6 Hb is expected.

The functional capillary density seen with EAF P3K6 Hb is comparable to that seen with EAF P5K6 Hb, however flow is better with EAF P3K6 Hb compared to EAF P5K6 Hb. Both microcirculatory parameters are significantly better than with dextran 70. Therefore, we conclude that EAF P3K6 Hb is also a supra plasma expander just as EAF P5K6 Hb.

In extreme hemodilution studies, the amount of Hb placed in plasma in our studies by using a 4 gm % solution of PEG Hb represents only about 20% of total Hb in circulation. This will be slightly less than 30% in EAF P3K6 Hb experiments where we have used 6 gm % solution to maintain a viscosity comparable to that of 4 gm % of EAF P5K6 Hb. In severe anemia where hematocrit is very low and tissue oxygenation by the RBC (through direct diffusion) is essentially minimal, high oxygen affinity PEG-Hbs indeed improves oxygen delivery to tissues. In the presence of MP4 there is a significant oxygen debt in the venular blood as compared to EAF P3K6 Hb. In MP4 increase in tissue oxygenation is minimal, while with EAF P3K6 Hb it is slightly better than P5K2 Hb. However, concentration of EAF P3K6

Hb in plasma is nearly 50% more than with P5K2 Hb, i.e., overall efficacy may not reach the level seen with P5K2 Hb. The improved oxygen delivery in the presence of these high oxygen affinity Hbs could be a direct consequence of improved microcirculation, or increased oxygen carrying capacity of the system, or a combination of both increased oxygen carrying capacity and improvements in the oxygen transfer capability from the oxy-Hb in the RBC to the tissues (i.e., catalytic transfer). These aspects of high oxygen therapeutics contributing to improved tissues oxygenation are yet to be sorted out. We believe that oxygen transfer catalytic activity of PEG-Hbs (nano oxygen-pumps) plays a dominant role in situations of treating severe anemia, as more clearly reflected in the experiments with transgenic sickle mice discussed below.

It may be noted that we had generated P3K2 Hb also to see how this smaller molecule can compare with P5K2 Hb. However, this PEG-Hb was cleared from the circulatory system and P3K2 Hb was seen in the urine and thus could possibly damage the kidney (nephrotoxicity). Notably P5K2 Hb is not cleared through kidney, the shorter PEG 3K apparently binds tightly to the Hb molecule providing a compact molecule while the larger PEG 5K chains have more disorder in their structure. This apparently contributes significantly to the larger hydrodynamic volume and attenuation of kidney filtration (Fig. 20.4).

Therapeutic Application of High Oxygen Affinity PEG Hb in Treating the Pathophysiology of Sickle Cell Disease

Sickle cell disease is the first molecular disease to be identified and is a consequence of the presence of mutant Hb, namely HbS. HbS polymerizes when deoxygenated in a HbS concentration dependent fashion. The sickled erythrocytes are deformed to various degrees and clog the capillaries leading to regional vaso-occlusion. However, adult sickle cell anemia is essentially a co-morbidity disease. Accordingly, therapeutic benefits may be derived from approaches besides those focused on inhibiting deoxy HbS polymerization. Carbon monoxide has been advanced for modulation of the vaso occlusive crisis. Sangart's MP4CO, carbon monoxy Hb has been approved as orphan drug; however, Sangart ceased its operation before they could initiate a phase II clinical trial. Prolong's Sanguinate, a carbonmonoxy Enzon PEG Hb has also been advanced as an alternate therapeutic to inhibit polymerization, and phase II studies have provided positive results.

Alternatively, our studies evaluating EAF P3K6 Hb and EAF P5K6 Alb as a therapeutic agent to treat sickle cell disease using transgenic sickle mice have focused on the potential applications of supra plasma expansion activity with and without oxygen carrying capacity, respectively, to treat severe anemias and particularly with a goal of targeted oxygenation of hypoxic tissues.

(i) *Supra Plasma Expanders Attenuate Hypoxia Reoxygenation Mediated Vaso-Occlusion in Transgenic Sickle mice NY1DD, EAF P3K6 Hb is superior to EAF P5K6 Alb both in NY1DD and Berk***:** We used three models of transgenic mice in our lab at Einstein for study of SCD; each model differs from one another in terms of the degree of anemia and severity of disease. The mildest transgenic mouse model is NY1DD. The S + S Antilles models is also a mild model which express only mild anemia and very limited vaso-occlusion just as NY1DD but its tolerance for hypoxia is less than that of NY1DD. Severe vaso-occlusive episodes are induced into these mild models by subjecting the animals to hypoxia reoxygenation. The NY1DD model tolerates long exposure to hypoxia reoxygenation while the model S + S Antilles can tolerate only shorter durations of hypoxia and reoxygenation. In NY1DD and S + S Antilles, the cerebral blood flow is essentially comparable to the wild type of mouse, whereas the Berkley (Berk) mouse exhibits significantly higher CBF nearly 2.5 to three times higher than the wild type mouse and is accompanied by severe anemia with hematocrit levels around 30.

Intaglietta and his colleagues [15] have shown that MP4 oxy improves the functional capillary density in the Alabama transgenic mouse models (which are mild disease model just as with NY1DD) of sickle cell disease when the animals are exposed to hypoxia reoxygenation. This reflects the potential protective role of supra plasma expanders in mitigating the hypoxia induced influence on the microcirculation in mild sickle cell disease situations.

The therapeutic activity of supra plasma expanders with and without oxygen carrying capacity (EAF P3K6 Hb and EAF P5K6 Alb) to attenuate hypoxia-reoxygenation induced vaso-occlusion in the NY1DD models has been studied at Einstein. Both supra plasma expanders attenuated the hypoxia reoxygenation induced vaso-occlusion as reflected by the clearing of vaso-occlusion in veins of treated mice in comparison to untreated mice. EAF P3K6 Hb was better than EAF P5K6-Alb in this regard. The pattern of improvement obtained with EAF P3K6 Hb in the NY1DD was excellent – treated mice exhibited improvement as good as the wild type controls. Interestingly, pretreating NY1DD with EAF P3K6Hb protects the mice from developing hypoxia reoxygenation induced vaso-occlusion [16]. The amount of EAF P3K6 Hb in the plasma amounts to less than 10% of Hb in the RBC, which can hardly be considered as increasing the oxygen carrying capacity of blood. Rather, the protective effect against hypoxia-reoxygenation is likely like that obtained in experimentally induced extreme hemodilution studies that produce severe anemia discussed earlier. Though the level of anemia in NY1DD is mild, the hypoxia protocol induces a severe anemia as oxygen carried to tissues will be severely diminished. Accordingly, we conclude that the protection

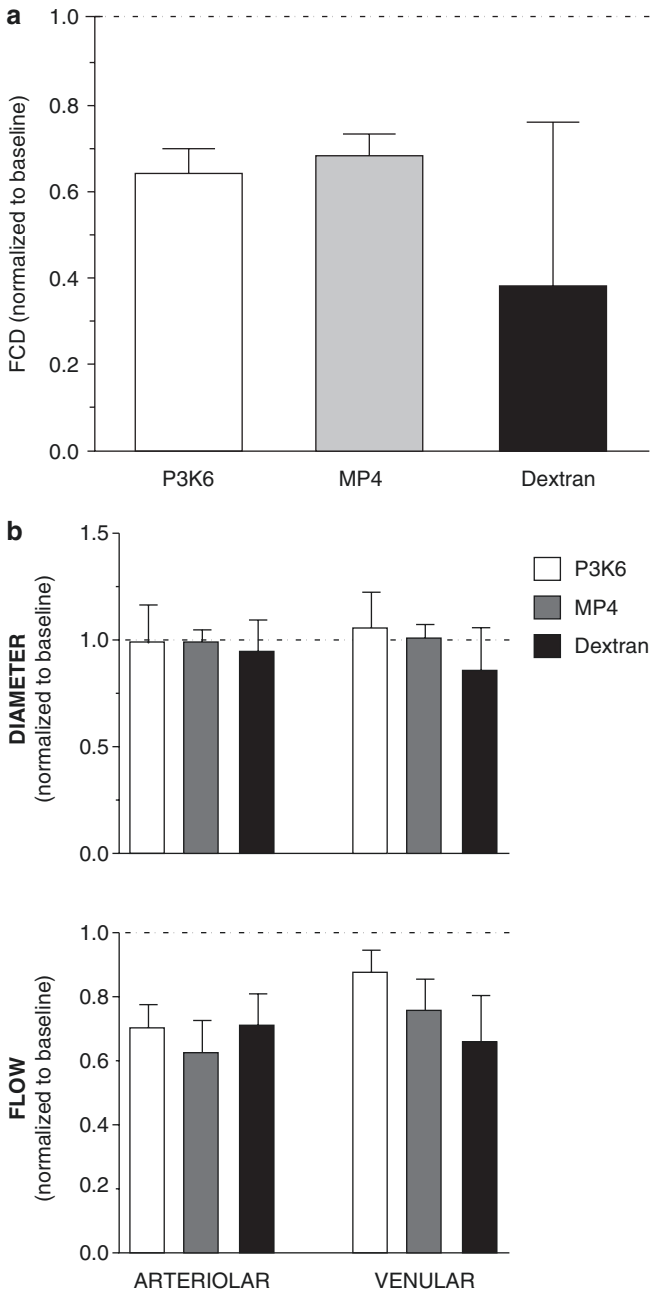


Fig. 20.4 (**a**) Influence of the mass of PEG-chain (size of PEG-shell) on Hb when it is surface decorated with EAF PXK6 pattern of PEGylation on microcirculation in extreme hemodilution studies in hamster as reflected by functional capillary density. X in PXK6 pattern, represents mass of the PEG-chains. (**b**) Influence of pattern of PEGylation of EAR PXK6 Hb, where as "X" represents the mass of PEG-chain used for surface decoration on blood vessel diameter and flow. The arterial diameter in all three materials. Venular diameter is essentially same as the base line but with dextran 70 it is slightly lower compared to PEGylated Hbs. The arterial and is nearly the same in all three samples, the venular flow seems to be best with EAF P3K6 Hb. (**c**) Influence of the mass of PEG-chain on Hb surface decorated in EAF PXKY pattern of PEGylation on oxygen distribution in arterioles, veins, and tissues in extreme hemodilution. Differences in Pattern of PEGylation on has essentially very limited influence on the PO2 in arterioles.as the P02 in the control dextran 70 samples is slightly less than the two Hb samples. Pattern of PEGylation on Hb impacts the venular PO2 levels. The PO2 level in EAF P3K6 Hb is comparable to that in control dextran sample are comparable while there is an oxygen debt in the MP4 sample. This suggests that as the blood has travelled from arterioles to veins, increased oxygen delivered to tissues has created an oxygen debt in the veins. Increased oxygen delivery (i.e., increased deoxygenation of oxy Hb as it travels through the capillaries to veins may be function of higher perfusion in the microcirculation due to the supra plasma expansion activity of MP4 or due to a combination of supra plasma expansion and increased oxygen extraction. Anyway, MP4 facilitates increased deoxygenation of oxy Hb. In dextran control there is not much decrease in oxy Hb in veins between EAF P3K6 Hb and dextran. Tissue oxygenation is best with EAF P3K6 Hb, and there is no difference in the tissue oxygen level in MP4 and dextran control. The difference in the tissue oxygenation level between that of dextran and MP4 is marginal. The decrease in venous blood should represent the metabolic consumption in MP4 treated animals

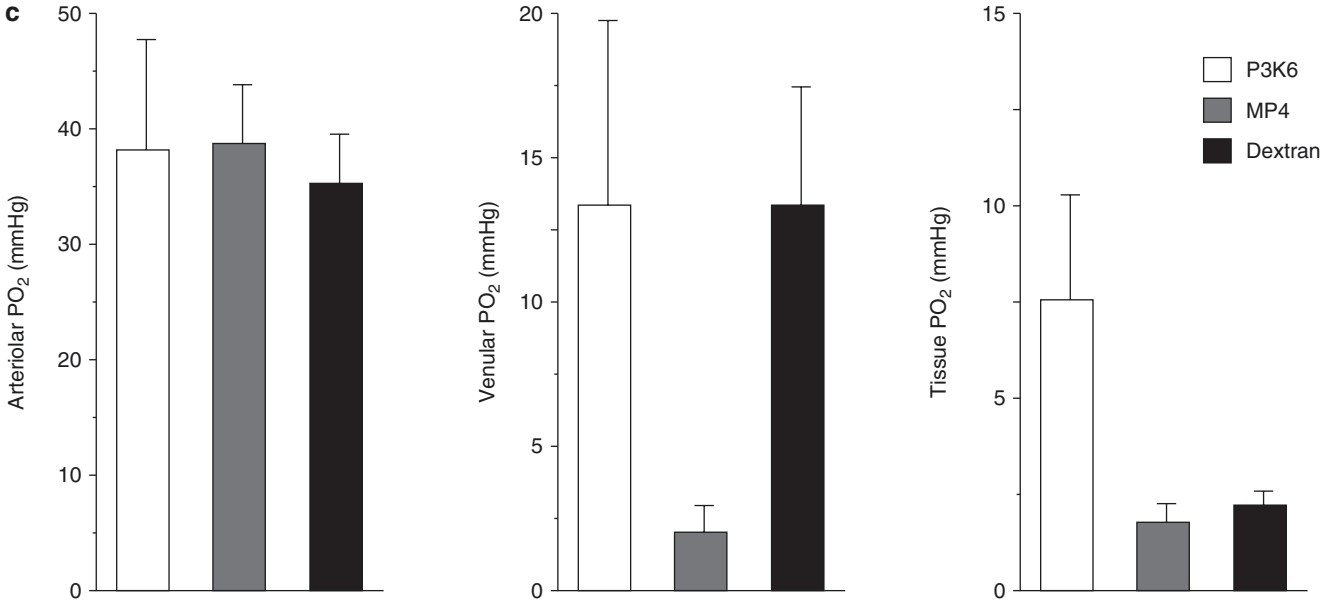


Fig. 20.4 (continued)

afforded by EAF P3K6 Hb represents essentially an improvement in the increase in the oxygen extraction from the RBC occurring during hypoxia. In the lungs, in a reverse way, EAF P3K6 Hb is expected to increase the oxygenation of the deoxy Hb in the RBC by facilitating the inflow of oxygen from alveolar space.

Berk is severe transgenic mouse model of sickle cell disease with severe anemia and intrinsic vaso-occlusion. Again, EAF P3K6 Hb is superior to EAF P5K6 Alb in attenuation of vaso-occlusion (Fig. 20.5). This reflects role oxygen delivery in attenuating the vaso-occlusion in these mice.

(ii) *Influence of Supra Plasma Expander EAF P5K6 Alb on Cerebral Blood Flow (CBF) in wild Type mice***:** We have demonstrated that the supra plasma expander EAF P5K6 Alb increases CBF after a 10% top load of the solution to the circulation. The increase in CBF was associated with a corresponding decrease in the level of deoxy Hb in the brain. This increase in CBF co-commitant with a decrease in de-oxy Hb is consistent with the supra plasma expansion activity seen in systemic microcirculation in extreme hemodilution studies (Fig. 20.6). It should be noted that in these studies, hematocrit was normal before and after administration of the compound and yet we still observed increased CBF. This finding is evidence of the supra plasma expansion activity obtained by top load of as little as 10% by volume of the test solution. Notably, the increase in CBF persisted for as long as 72 hours after the EAF P5K6 Alb infusion (Fig. 20.6). The 'luxury perfusion' induced by the supra plasma expander decreases the deoxy Hb content in blood.

(iii) *Influence of the severity of the sickle cell disease on the Cerebral Blood Flow (CBF) of transgenic sickle mice:* High CBF and increase in stroke represents a comorbidity of the adult sickle cell patients. We have compared the CBF in all three transgenic mouse models of sickle cell disease with that in wild type mice. In transgenic sickle mice there is an increase in CBF, (Fig. 20.7) and it corresponds to the severity of anemia just as the severity of the disease as represented by level of vaso-occlusion in steady state. The higher CBF shows a correspondence with levels of deoxy Hb in blood.

(iv) *EAF P5K6 Alb, Supra Plasma Expander without Oxygen Carrying Capacity, induces Oxygen Debt in the Brian in Transgenic sickle mice Berk:* Wild Type (WT) C57BL6 mice and three models of sickle cell disease (the mild NY1DD and Antilles models (not shown) and the severely anemic Berk model) underwent MRI assessment for CBF at 4 time points prior to and after administration of a 10% top load of EAF-P5K6-Alb, administered by tail vein. CBF was measured using arterial spin labeling methods, and brain tissue deoxy-Hb levels were assessed by blood oxygen level dependent changes observed before and after administration of 100% O2 [17] which is sensitive to the change in deoxy Hb in the venous effluent from the brain tissue. After the top load of EAF P5K6 Alb, CBF increased progressively, reaching maximal levels after 72 hours. In the NY1DD and Antilles animals, CBF remained

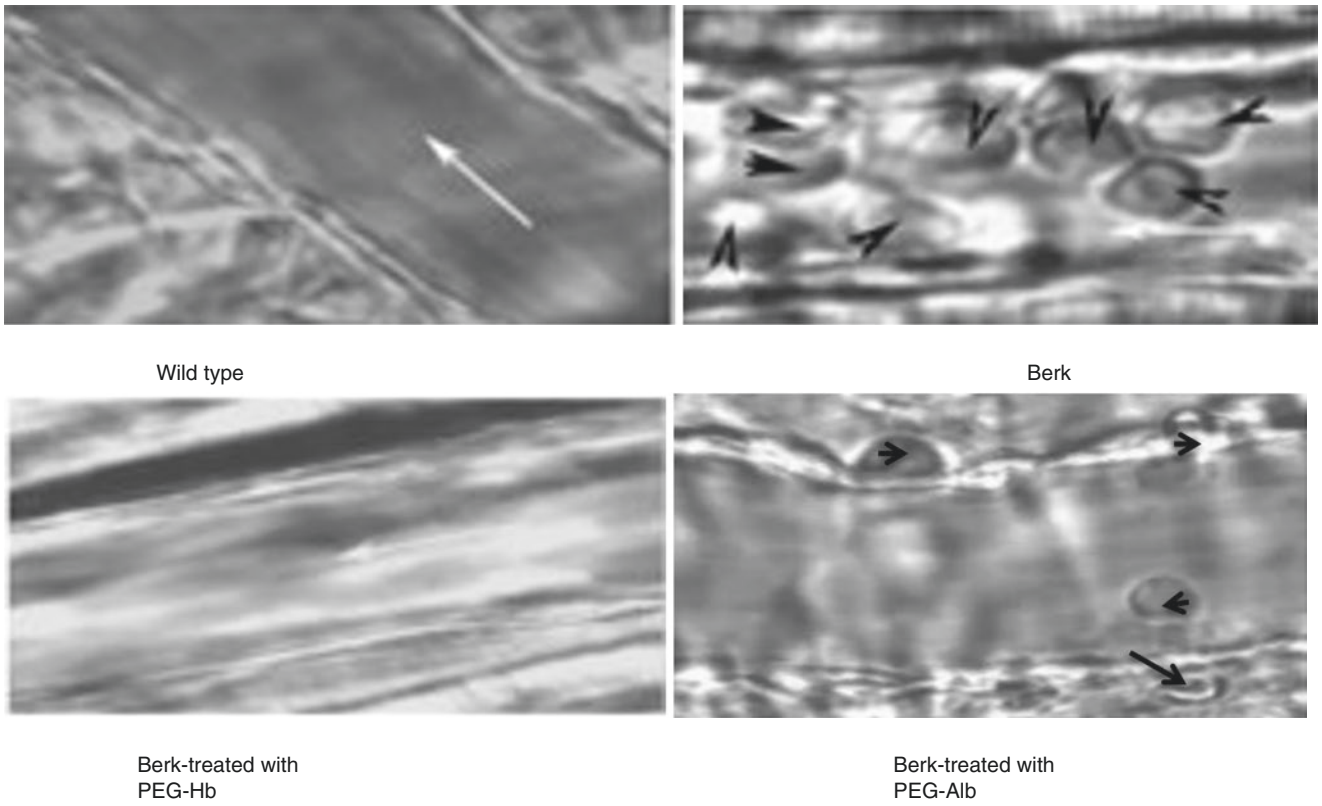


Fig. 20.5 Comparison of the video micrographs of veins of Berk mouse compared with wild type, before and after treating with EAF P3K6 Hb and EAF P5K6 Alb. Superiority of EAF P3K6 Hb can be seen from these studies

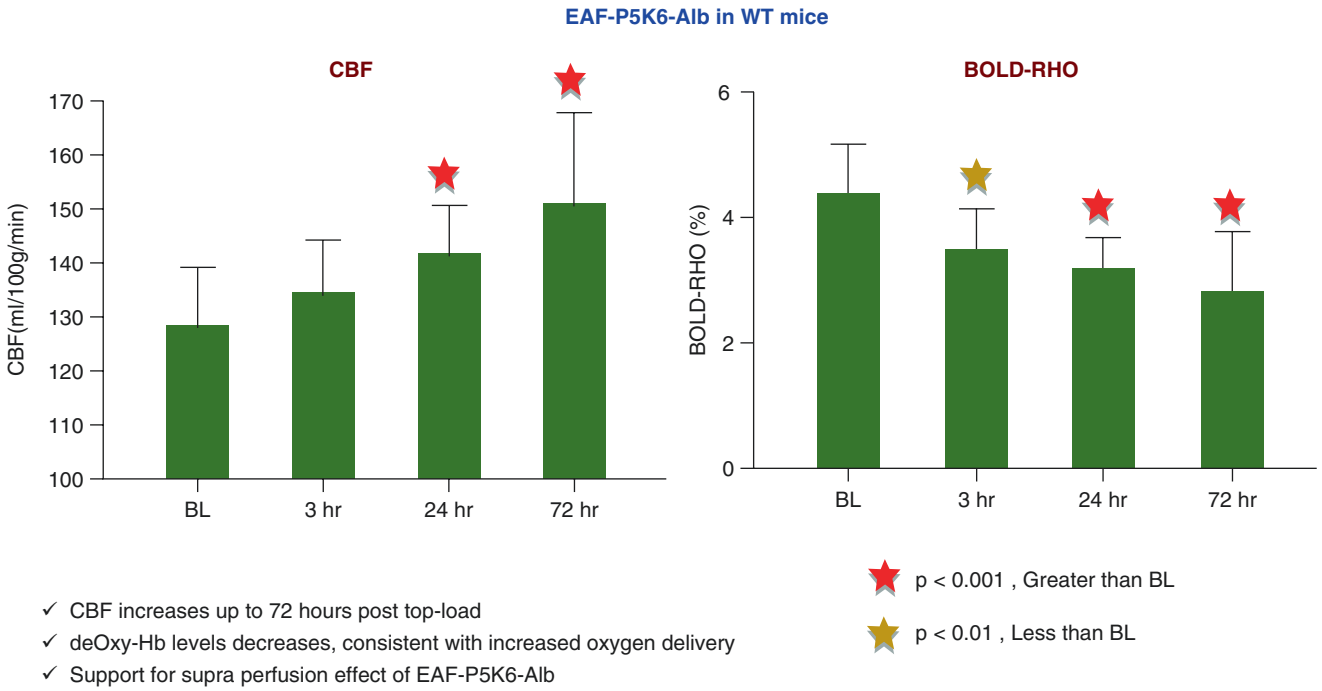


Fig. 20.6 Influence of top loading of EAF P5K6 Hb (10 %by volume) in wild type mice on cerebral blood flow (CBF) and deoxy Hb content in the blood as a function of time. CBF increases up to 72 hours after infusion of the test solution, in spite of the fact these mice of normal hematocrit. With the increase in blood flow, the amount of deoxy Hb in blood is reduced, consistent with a "luxury perfusion"

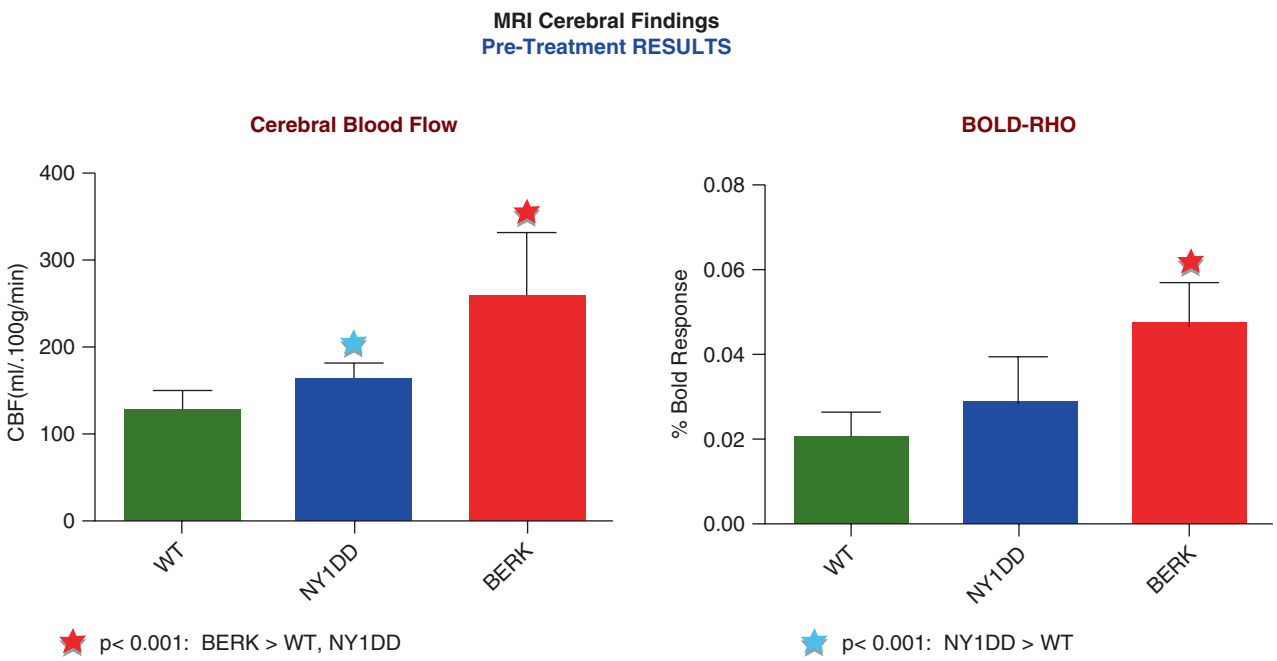


Fig. 20.7 Cerebral blood flow in transgenic sickle mice is a correlate of the severity of the disease. In transgenic mice, small but noticeable increase of CBF is seen. In S+ S Antilles, the CBF is nearly comparable to that of NY1DD. In Berk, severe disease model, CBF increased significantly. The increase in CBF is accompanied by corresponding decrease in deoxy Hb levels in blood

essentially unchanged at 24 hours or 72 hours. It may be noted that in these mice systemic circulation does not responds much to vaso-dilator, sodium nitroprusside, whereas the WT mice respond readily to vaso-dilators.

Surprisingly, Berk animals responded with decreases in CBF after top load of EAF P5K6 Alb. In Berk animals, anemia has driven the CBF significantly above WT levels, to facilitate an increase in oxygen delivery to meet cerebral metabolic demand in the face of inadequate oxygen carrying capacity. These CBF changes are confirmed in the deoxy-Hb measurements (sight side).

Increasing CBF in WT animals was associated with a concomitant increase in the efficacy of oxygen extraction in tissues and more efficient (frequent) reoxygenation of deoxy-Hb in lungs, resulting in decreased deoxy-Hb in the venous blood. In NY1DD animals, no change in CBF was reflected in no change in deoxy-Hb levels but the therapeutic benefit of EAF P5K6 Alb in attenuation of the hypoxia reoxygenation induced vaso-occlusion in systemic circulation has been demonstrated. The response of cerebral circulation in anemic BERK mice is very distinct as compared to WT or NY1DD. In BERK animals there is small drop in CBF on infusion of EAF P5K6 Alb. This reduction in CBF induced an increase in venous deoxy-Hb levels, suggesting further metabolic impairment. While the findings in the WT animals are interpreted to reflect the supra plasma expansion activity of the EAF P5K6 Alb, the decrease in Berk is likely resulting from an impaired CBF regulatory system in which nitric oxide responsiveness is impaired from NOS scavenging of free Hb from RBC lysis. It may be noted that Berk mice exhibit intrinsic high vaso-occlusion in systemic circulation, and it is cleared significantly cleared by EAF P5K6 Hb. Accordingly, this result suggests the need for SCD therapeutic compounds that improve the systemic circulation by attenuating the vaso-occlusion should be coupled with strategies that provide additional oxygen to hypoxic tissues like brain and kidney, particularly in disease situations with severe anemia.

(v) *EAF P3K6 Hb, a Supra Plasma Expander that Binds Oxygen Reversibly, Normalizes CBF in Berk without*

Creating an Oxygen Debt: Infusion of EAF P3K6 Hb to NYIDD prior to submitting the animals to hypoxia reoxygenation platform, protects these transgenic mice developing severe vaso-occlusion (Feng et al). Besides, EAF P3K6 Hb clears the systemic vaso-occlusion completely in Berk, and significantly better than EAF P5K6 Hb.

To map the influence of supra plasma expanders with and without oxygen carrying capacity on the cerebrovascular system, EAF P3K6 Hb was administered to WT and Sickle mice, and the response compared to the response after EAF P5K6 Alb, which does not carry Hb. In the WT and NY1DD mice there were no significant changes in either CBF or deoxy-Hb, except for a short-term slight reduction in CBF right after the top load was given for which no change in deoxy-Hb was observed. However, in severely anemic Berk mice the response was quite different, as illustrated here. While the top load of EAF P5K6 Alb induced a slight decrease in CBF which increased deoxy-Hb levels, the top load of EAF P3K6 Hb transiently decreased CBF but they rebounded above pre-top load levels by 72 hours. Importantly, the deoxy-Hb levels remained unchanged from pre-top load levels throughout these changes, suggesting that the EAF P3K6 Hb improved the oxygen extraction even when delivery was reduced by reduced CBF. This effect must be mediated by increased oxygen carrying capacity and a targeted delivery to the most hypoxic tissues. Thus, the EAF P5K6 Hb was protective against the decrease in CBF and maintained normal oxygen tension in the tissue.

Novel Aspects of EAF PEGylated Albumin and EAF PEG-Hb as Oxygen Therapeutics

Both EAF PEGylated Alb and EAF PEGylated Hb may be considered as novel oxygen therapeutics that help us to modulate oxygen delivery without significantly altering the oxygen delivering capacity of the circulatory system. These supra plasma expanders can be considered as novel nanoparticles that are endowed with RBC like biophysical property to modulate the microcirculation, and as such are unique molecular structures. These are nanostructures only from the perspective of their hydrodynamic volume and not by their mass. EAF PEG-Alb, one without any oxygen carrying capacity improves microcirculation in situations when the hematocrit levels are low (about 25% of the original) without significantly improving the tissue oxygen levels (extreme hemodilution). Even when hematocrit is normal, the supra plasma expansion properties increase the CBF and lowering the deoxy Hb levels in blood much like a vasodilator without apparent hypotensive effects. It may be noted that, we have previously suggested that EAF PEG Alb can be used in place of small amounts of blood for transfusion; this is possible since EAF PEG Alb improves oxygen extraction by improving functional capillary density and blood flow. It may be noted human body is designed to use just about 20% of the overall oxygen carrying capacity of the circulatory system. This redundancy in the oxygen carrying capacity vs the real need for the metabolic reaction allows us to minimize the use of blood transfusion when blood loss or anemia is marginal; if we can find a better way of improving the oxygen extraction when there is blood loss or anemia. This is a totally novel concept and practical application of these concepts requires further studies and undertaking of the clinical trials.

Improved oxygen extraction in the presence of supra plasma expander appears to be a function of the reversible oxygen binding of EAF P3K6 Hb in plasma in the circulatory system. The resolution of hypoxia reoxygenation induced vaso-occlusion in NY1DD (low anemia sickle cell model) is accomplished quicker in the presence of EAF PEG Hb than in its absence. On the other hand, EAF P3K6 Hb is better even when anemia is present and is in the range of transfusion trigger, i.e., severe as in Berk.

However, when severe anemia is present as in Berk (with Hb levels around 7– gm/dl), EAF P5K6 Alb creates an oxygen debt in the brain, even though we could see significant therapeutic benefit in terms of attenuating vaso-occlusion in systemic circulation. On the other hand, EAF P3K6 Hb normalizes CBF and provides protection against the by normalizing CBF in maintaining oxygen saturation in the blood. This is apparently accomplished by improved extraction from RBC, thus avoiding the creation of transient oxygen debt.

We believe that the therapeutic activity of EAF P3K6 Hb in BERK mice, particularly in normalizing the CBF without increasing the deoxy Hb content in the venous blood, cannot be attributed simply to increased oxygen carrying capacity afforded by EAF P3K6 Hb placed in plasma. The high oxygen affinity PEG Hb in the plasma accounts for less than 10% of total Hb in the circulatory system (Hb in RBC + EAF P3K6 Hb in the plasma). It may be noted that normalization of CBF in Berk by fetal Hb, requires the Hb content in blood to be around 11–12 gm/dl. This implies that the efficacy of EAF P3K6 Hb placed in plasma is nearly onefold higher than fetal Hb in the RBC. This is counter intuitive to our present understanding of delivery of oxygen by Hb, increase the oxygen carrying capacity to compensate for the reduction in hematocrit (oxygen carrying capacity).

Accordingly, we suggest the high oxygen affinity of EAF P3K6 Hb placed in plasma facilitates the transfer of oxygen from RBC by a "pull" mechanism to maintain a steady-state level of oxygen tension in plasma and this facilitates the diffusion of oxygen to the tissues. The supra plasma expansion activity of EAF P3K6 Hb, particularly, the increased functional capillary density facilitates a better diffusion of oxygen from plasma to tissues. PEG Hb in plasma acts as a nanomachine (catalysts) that facilitates more out-flow of oxygen from RBC to plasma (nano oxygen-pump). The increased oxygen tension in plasma due to the presence of EAF P3K6 Hb facilitates the diffusion (push) of oxygen to tissues thereby facilitating an increase in the delivery of oxygen to the tissues. The concepts of supra plasma expansion by low viscosity colloidal plasma expanders and catalysis of oxygen transfer by high oxygen affinity Hb are new concepts. The synergistic combination of these two in EAF PEG Hb provides a new opportunity to design new-found members of this novel class of molecules.

In severely anemic Berk mice, increasing the oxygen extraction from RBC is expected to increase the concentration deoxy HbS in the circulation without a compensation for increasing the oxygen carrying capacity of existing RBC, Increased oxygen extraction from RBC is a polymerization promoting process. However, we have not seen any evidence of that with Berk mice and deoxy HbS levels in the brain is essentially remains unchanged even when CBF has been pretty much normalized to wild type levels. Accordingly, we hypothesize that in the presence of EAF P3K6 Hb, there is an increase in the oxygen saturation of HbS in RBC in the lungs, i.e., an increase in the oxygen carrying capacity blood.

It is generally known that P50 of RBC in HbSS patients is around 35 mm Hg whereas the P50 of RBC from HbAA individuals is around 28 mm Hg. However, the intrinsic oxygen affinity of HbA *in vitro* is indistinguishable from that of HbS. 0xygen saturation levels in normal individuals is around 99% while that with SCD patients is around 95%. The lower oxygen affinity of RBC in SCD patients reflects the problem associated with inflammation in lungs and the presence of small deoxy HbS polymers in the RBC. The presence of high oxygen affinity EAF P3K6 Hb in the plasma, apparently, facilitates the in-flow of oxygen to RBC from the alveolar space in the lungs (a reverse of what happens in the tissues). The presence of EAF P3K6 Hb increases the oxygen tension in the plasma higher as compared to its absence, this facilitates the oxygenation of deoxy HbS in lungs better than in the control animals. This likely leads to the depolymerization of some deoxy HbS polymers in the RBC, thus adding to the increased oxygen carrying capacity of the blood. It may be noted here oxygen therapy is being advanced as a novel strategy to treat painful vaso-occlusive crisis in SCD patients and this is to increase the oxygen saturation in patients with sickle cell disease [16, 17].

The SCD therapeutic activity of EAF P3K6 Hb to attenuate hypoxia-reoxygenation induced vaso-occlusion in NY1DD as well as the normalization of CBF in Berk without any changes in deoxy HbS content in the veins, suggests a potential application of EAF P3K6 Hb as an alternate to oxygen therapy in emergency medicine and critical care medicine. Oxygen therapy is standard platform to treat pulmonary complications due to insufficient oxygenation of Hb in lungs, including but not limited to Covid-19. Low venous oxygenation levels are equivalent to hypoxia induced low saturation of Hb in blood. Our experience with EAF P3K6 Hb in transgenic sickle mice opens this approach for treating situation where there is a drop in oxygen saturation levels requiring oxygen therapy (Fig. 20.8).

Increasing oxygen affinity of Hb in RBC has been advanced as a therapy to treat Covid-19, hydroxymethyl furfural has been advanced as a potential therapeutic for this purpose [18]. GBT-440 [19], approved as an emergency medicine for sickle cell disease, is probably a better therapeutic to increase the oxygen affinity of Hb *in vivo*. Though there appears to be little doubt in terms of therapeutic benefit of GBT-440 in sickle cell disease, the mechanism of action of the therapy has been debated. Increasing the oxygen affinity of Hb has been suggested to increase the anemia effect in sickle cell anemia patients [20] just as increasing the oxygen extraction using supra plasma expanders. On the other hand, just as with high oxygen affinity oxygen therapeutic EAF P3K6 Hb, the high oxygen affinity RBC could also accomplish a targeted oxygenation of hypoxic areas of body in sickle cell patients. Similarly, high oxygen affinity RBCs can increase saturation of oxygen in lungs thereby contributing to overall therapeutic benefits. High oxygen affinity RBC generated *in situ* could also lead to increased oxygen saturation in lungs as well. High oxygen affinity fetal Hb has been demonstrated to accomplish peripheral oxygen saturation [20]. Increasing the oxygen affinity of RBC has also been shown to improve microcirculation and better tissue oxygenation [21]. An Interesting situation could be a combination of high oxygen affinity inducing GBT 440 along with EAF P3K6 Hb in treating severe sickle cell patients as well as patients with pulmonary complications with reduced oxygen saturation levels. In this respect, of particular interest, is covid-19 patients with severely low levels of oxygen saturation.

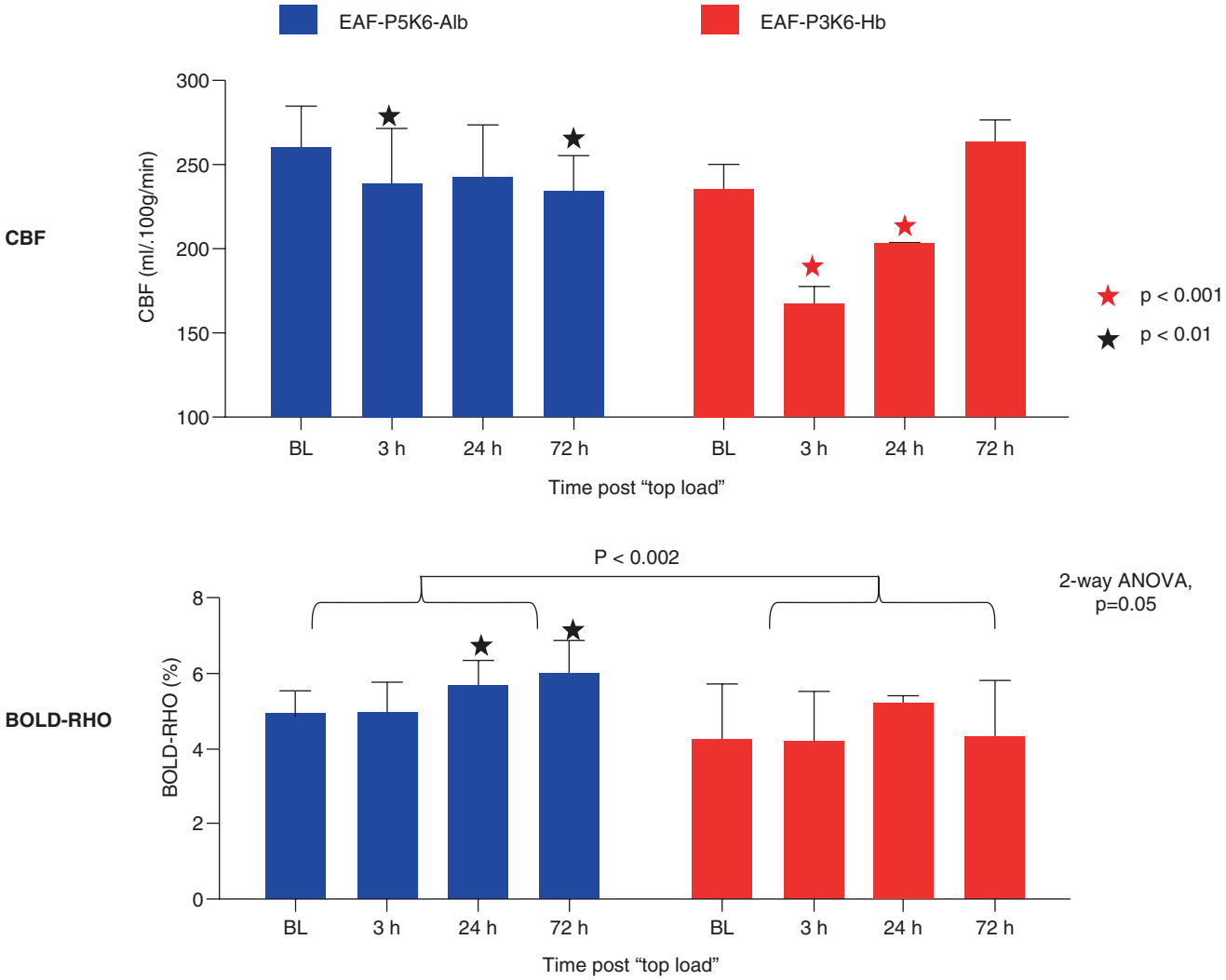


Fig. 20.8 Comparison of the influence of top loading transgenic sickle mice Berk with EAF P5K6 Alb and EAF P3K6 Hb on brain CBF and deoxy Hb levels. Hb levels in Berk mice is around 7–8 gm %, and hence are very severe. Berk mice do not show the supra perfusion seen with wild type, i.e., the system tuned up as much as possible. None the less we can see some lowering of the high CBF Berk mice, a consequence severe sickle cell disease with severe anemia. Even though EAF P5K6 Alb clears the systemic vaso-occlusion noticeably, it does very little to normalize the CBF and is accompanied by creating an oxygen debt in the brain. EAF P3K6 Hb, normalizes the CBF in 3 hours without creating an oxygen debt in the brain, and gets back to pre-infusion level by 72 hour

General Conclusions

In this chapter we have refocused the issues one needs to consider when designing high oxygen affinity Hb as a new class of oxygen therapeutics, particularly the influenced engineered modifications on on- and off-rates of oxygen form Hb and the role of the PEG shell in dictating the outcome when administered into animals. Our studies have established high oxygen affinity EAF P3K6 Hb is more efficient relative to EAF P5K6 Hb and MP4. The anti-anemia therapeutic efficacy as reflected from the studies with transgenic sickle mice is significantly higher than increasing the hematocrit level (transfusion). Surprisingly, this efficacy is superior to generating high oxygen affinity RBC *in vivo* either by chemical or genetic approaches as well; we calculate this efficacy as reflected by the normalization of CBF is nearly an order of magnitude better. Accordingly, a new concept has been advanced here: the efficacy of SCD therapeutic activity of high oxygen affinity EAF P3K6 Hb placed in plasma in transgenic sickle mice stems from a catalyst- like activity of EAF P3K6 Hb, a nano oxygen pump. This activity also facilitates an in-flow of oxygen to RBC in lungs besides the out-flow of oxygen from RBC to tissues in rest of the body. As such, the newly designed oxygen therapeutic is used in very small amounts as compared to Hb inside RBC (less than 10%).

These results are counter intuitive to current paradigm of designing blood substitutes which focus on increasing the oxygen carrying capacity of circulatory system. In the design of EAF P3K6 Hb and EAF P5K6 Hb, a new approach to design semisynthetic supra plasma expander has been exposed. The design of EAF P5K6 Alb, a non-oxygen carrying semisynthetic supra plasma expander confirms this activity is independent of oxygen binding/carrying capacity. However, we need to recognize that supra plasma expansion by EAF P5K6 Hb and EAF P5K6 Alb may be very distinct and may be a function of the efficacy of release of oxygen from RBC. It may be noted that release of oxygen from oxy Hb in RBC will also release NO, the vasodilator [21]. Thus the targeted oxygenation of tissues by high oxygen affinity PEG-Hb also accomplishes a targeted release of Nitric oxide, or targeted vasodilation. This is independent of the intrinsic supra plasma expansion activity engineered by EAF hexa-PEGylation. Thus, with EAF P3K6 Hb and EAF P5K6 Hb, a better perfusion could be expected with PEG Hbs relative to EAF P5K6 Alb through a synergy of improved shear thinning (supra plasma expansion) and release of NO through improved oxygen extraction of oxygen (deoxygenation) inside RBC. Supra plasma expansion by EAF P5K6 Alb operates primarily through increased shear thinning of blood and endothelial NO production. Increased deoxygenation of oxyHb inside RBC in the presence of high oxygen affinity PEG-Hb increases the flexibility of RBCs. Besides, with the newly designed PEG Hbs the low-level use of oxygen therapeutic is expected to attenuate the toxicity associated with the development of blood substituted as oxygen carrying materials on the one hand and the cost of the treating patients on the other.

Our initial thoughts in the design of high oxygen affinity EAF P5K6 Hb has been to target the oxygenation of hypoxic tissues, where the oxygen demand is high. EAF P3K6 Hb is essentially devoid of any Bohr effect. Accordingly, the in-flow and out-flow of oxygen to RBC in lungs and to tissues, respectively, is enhanced during acidosis essentially as a linear function of lowered pH induced by anemia/hypoxia. As the pH is lowered, the affinity oxygen to Hb inside RBC is lowered, i.e., the difference in the affinities of Hb inside RBC and EAF P3K6 Hb increases. The net oxygen-pull power of EAF P3K6 Hb increase in hypoxic tissues as a function of acidosis. Application of high oxygen affinity oxygen therapeutics as molecular pump placed in plasma to increase oxygen extraction from RBC that synergizes the high oxygen affinity PEG-Hbs with its supra plasma expansion activity induced by EAF PEGylation for treating anemia or blood loss, represents a clear paradigm shift for designing novel class of a oxygen therapeutics.

Future Directions

It may be noted here that microcirculatory parameters of P5K2 Hb are better than of EAF P3K6 Hb. Concomitant with this, the relative efficacies of tissue oxygenation of P5K2 Hb is better than with EAF P5K2 Hb (Table 20.3). The relative efficacies of some of the PEG Hbs studied by us is summarized in Table 20.3 and compared with that of MP4 and MP8, suggesting that tissue oxygenation is not a direct correlate oxygen affinity, but better tissue oxygenation is generally associated with better microcirculatory parameters. The data in Table 20.3 suggest di-PEGylated Hb is a better 'nano oxygen pump' compared to hexa-PEGylated Hb. We anticipate that the shear thinning is probably maximum with EAF P3K6 Hb and EAF P5K6 Hb, supra plasma expanders and hence expected to provide a better microcirculation. Therefore, higher efficacy of tissue oxygenation with P5K2 Hb is indeed surprising and suggest that as the efficacy of oxygen extraction from RBC increases, the concomitant release of NO plays a more important role in providing better microcirculatory parameters than the hexa-PEGylation induced supra plasma expansion.

Improving the efficacy of tissue oxygenation using small amounts of high oxygen affinity Hb placed in plasma through better oxygen extraction from RBC is a novel concept advanced here. The transfer of oxygen from oxy Hb in RBC to tissues is marginal even with supra plasma expander like EAF P5K6 Alb or dextran 500, i.e., diffusion mediated transfer of oxygen from through plasma when the hematocrit is low is negligible. The increase in the transfer of oxygen in the presence of high oxygen affinity Hb could be accomplished either through improved oxygen tension in the plasma (established through reversible equilibrium or through facilitated transport of oxygen by PEG-Hbs in the plasma. The release of vasodilator by the deoxygenation of oxy Hb inside RBC appears add significantly more improvement in microcirculation than that achieved through supra plasma expansion. The difference between EAF P3K6 Hb and EAF P5K6 Hb in tissue oxygenation certainly empha-

Table 20.3 Relative efficacies of tissue oxygenation by PEGylated Hbs

	Relative efficacy of tissue oxygenation
PEGylated sample	Tissue PO2/Conc of Hb in plasma
P5K2 Hb	7.8
P10K2 Hb	8.0
P5K4 canine Hb	4.0
P5K4 rHb(αH20C)	5.6
EAF P5K6 Hb (Einstein)	3.0
MP4 (EAF P5K8 Hb)	1.6
MP8 (EAF P5K8 Hb)	2.5
EAF P3K6 Hb	5.1

sizes the relative roles of on and rates of interaction of oxygen with PEG Hb.

Though Hb EAF hexa-PEGylated with maleimide PEG-5K was chosen initially because this represented the maximum number PEG-5K chains that can be accommodated on the molecular surface of Hb without perturbing interdimeric interactions of PEG-Hb. However, higher number of PEG-chains seems to attenuate the efficacy of oxygen extraction RBC and thus tissue oxygenation by high oxygen affinity Hbs. Lowering the mass of PEG-chains conjugated to Hb in hexa-PEGylated shows improvement in tissues oxygenation. In doing we have also reduced the molecular dimensions of hexa-PEGylated Hb. It may be noted that there is generally an increase in the efficacy of tissue oxygenation as the molecular dimensions, i.e., hydrodynamic volume of Hb is reduced. As the molecular dimensions are reduced, the viscosity of the plasma layer is also reduced and this impact the movement of PEG Hb in the plasma layer.

It has been noted earlier that oxygen therapeutics deliver oxygen more efficiently than the Hb inside RBC and this has been attributed to the facilitated transport of oxygen by Hb in plasma as compared to the diffusion of oxygen through plasma layer. We believe that this is the case with high oxygen affinity PEG-Hb as well and the inverse correlation of relative efficacy of PEG-Hb mediated improvements in tissue oxygenation as a function of the molecular dimension of PEG-Hb suggests that there may be room to further improve the efficacy of these 'nano oxygen pumps' either through the manipulation of the off rates of oxygen from oxy-PEG Hb or through manipulation of the packing density of PEG-shell.

We have generated P5K2 αα-Hb as way of stabilizing the deoxy conformation of P5K2 Hb molecule, which increases the P50 of the molecule. Preliminary studies show P5K2 αα-Hb retains better microcirculatory properties as compared to EAF P3K6 Hb, but not as effective as P5K2 Hb. The efficacy of tissue oxygenation is more comparable to that of EAF P3K6 Hb. The oxygen affinity of P5K2 αα-Hb is around 12 mm Hg, i.e., lower than that of P5K2 Hb. The lower P50 reflects the easy access of deoxy conformational state for this molecule compared to P5K2 Hb due the presence of αα-intramolecular crosslink. This presumably facilitates an early release of oxygen in plasma and thereby lowers the efficacy of this molecule as "nano oxygen-pump" even though the lower molecular mass and lower plasma viscosity with P5K2 αα-Hb as compared to EAF P3K6 Hb should increases its efficacy as 'nano oxygen pump'. Accordingly, both structural aspects contribute to the efficacy of high oxygen affinity PEG-Hbs to facilitate better tissue oxygenation. The slight increase in the oxygen affinity of Hb from that of P5K2 Hb to that of EAF P3K6 Hb may have contributed to the attenuation the microcirculatory benefits seen in extreme hemodilution with P5K2 Hb.

Accordingly, we need to ask a new question: (i) can we increase the packing density of the high oxygen affinity Hb by generating non-PEGylated high oxygen affinity Hb and (ii) then can we synergize the supra plasma expansion activity of EAF P5K6 Alb with a high oxygen affinity non-PEGylated Hb, for example αα-Hb modified at its Cys-93(β) with a maleimide or alternated oligomerized αα-Hb that has been generated using new oligomerization platform, extension arm facilitated short bis PEG-maleimide mediated oligomerization. The latter platform introduces only intermolecular crosslinks, on an average, one intermolecular crosslink per tetramer through Cys-93(β). This product has molecular dimensions comparable to EAF P5K6 Hb but has a compact packing density essentially same as Hb. The product is a high oxygen affinity polymeric Hb with an oxygen affinity around 12 mm Hg; the lower oxygen affinity stemming from the stabilization deoxy like structure of Hb by αα-fumaryl cross bridge and this could provide an insight into the lower efficacy of tissue oxygenation EAF P3K6 Hb as compared to P5K2 Hb. Similarly, αα-Hb could be dimerized using Bis Mal-PEG-600 and the second Cys-93(β) modified with N-ethyl maleimide, this will have molecular dimensions and oxygen affinity comparable to P5K2 Hb. A study of tissue oxygenation by these can help us to map the relative roles of facilitated transport of oxygen and diffusion mediated transport of oxygen.

Finally, though both EAF P5K6 Alb and EAF P3K6 Hb are supra plasma expanders, the response of cerebral circulation to these two supra plasma expander is very distinct as seen with wild type mice. EAF P3K6 Hb, does not show an increase in CBF, in fact a small decrease during early times and it reverses to higher values with longer times. In wild type mice the response to EAF P3K6 Hb is short lived as compared to EAF P5K6 Alb. We believe that this reflects the differential stability of these two semisynthetic proteins and coupled with high oxidative stress in transgenic sickle mice. Presumably, the subunit structure of Hb and the potential dissociation of the tetrameric structure into αβ-dimes contributes to this instability. Alb is a single polypeptide chain even though molecular mass of the two proteins is essentially the same. Intramolecularly crosslinked αα-Hb could be used to generate EAF P3K6 Hb to overcome the limitations and then the therapeutic efficacy can be considered with P5K2 αα-Hb to see when the normalization effect could last longer with these molecular species. Transgenic sickle mice, particularly Berk is a good system to evaluate/establish many new concepts delineated in this chapter for designing new class of oxygen therapeutics.

Key Points

High oxygen affinity oxygen therapeutic, EAF PEG-Hb, supra plasma expansion, Nano-oxygen pumps, on and off loading of oxygen to RBC, sickle cell disease, semisynthetic

supra plasma expanders, oxygen therapy, high oxygen affinity RBC.

References

1. Jansen FB. The dual role of red blood cells in tissue oxygen delivery: oxygen carriers and regulators of blood flow. J Exp Biol. 2009;212:3387–93.
2. Acharya AS, Manjula BN, Smith P. 1996. Hemoglobin crosslinkers. US Patent 5585484.
3. Manjula BN, Tsai AG, Intaglietta M, et al. Conjugation of multiple copies of polyethylene glycol to hemoglobin facilitated by thiolation: influence on hemoglobin structure and function. Protein J. 2005;24:433–63.
4. Li D, Manjula BN, Acharya AS. Extension arm facilitated PEGylation of hemoglobin: correlation of properties with the extent of PEGylation. Protein J. 2006;25(4):263–74.
5. Ananda K, Acharya AS. Role of extension arm in PEG-Hb conjugates on the Stability of Tetramer: Nonconservative EAF maleimide thiol-PEG mediated PEGylation. Artif Cells Blood Substit Biotechnol. 2008;36:499–512.
6. Li D, Hu T, Manjula BN, et al. Non-conservative surface decoration of hemoglobin: influence of neutralization of positive charges at PEGylation sites. Biochem Biophys Acta. 2008;1784:1395–401.
7. Li D, Hu T, Manjula BN, et al. Extension arm facilitated PEGylation of alpha-alpha hemoglobin with modifications exclusively targeted to amino groups: functional and structural advantages of free Cys-93(beta)in PEG-Hb adduct. Bioconjug Chem. 2009;20:2062–70.
8. Winslow RM. MP4, a nonvasoactive polyethylene glycol-hemoglobin conjugate. Artfi Organs. 2004;28:800–6.
9. Vandegriff KD, Malavalli K, Wooldridge J. MP4, a new nonvasoactive PEG-Hb conjugate. Transfusion. 2003;43:509–16.
10. Sriram K, Tsai TG, Cabrales P, et al. PEG-albumin supra plasma expansion is due to increased vessel wall shear stress induced blood viscosity shear thinning. Am J Physiol Heart Circ Physiol. 2012;302:H2489–97.
11. Manjula BN, Tsai AG, Upadhya R. Site-specific PEGylation of hemoglobin at Cys-93(β): correlation between the colligative properties of PEGylated protein and the length of the conjugated PEG-chain. Bioconjug Chem. 2003;14:464–72.
12. Cabrales P, Kanika N, Manjula BN, et al. Microvascular P02 during extreme hemodilution with hemoglobin site specifically PEGylated at Cys-93(β) in hamster window chamber. Am J Physiol Heart Circ Physiol. 2004;287:11609–17.
13. Juszazak LJ, Manjula BN, Bonaventura C. UV resonance Raman study of β-93-modified hemoglobin A: chemical modifier-specific effects and added influence of polyethylene glycol chains. Biochemistry. 2002;41:376–85.
14. Khan ID, Dansker U, Samuni A, et al. Cys-93(β) modified hemoglobin: kinetic and conformational consequences. Biochemistry. 2001;40:7581–75922.
15. Tsai AG, Cabrales P, Young MA, et al. Effect of oxygenated polyethylene glycol decorated hemoglobin on microvascular diameter and functional capillary density in transgenic mouse model of sickle cell disease. Artfi Nanomed Biotechnol. 2015;43:10–7.
16. Meng F, Kaul D, Thangswamy S, et al. Semisynthetic supra plasma expanders: a new class of therapeutics to improve microcirculation in sickle cell anemia. Artfi Cells Nanomed Biotechnol. 2019;47:73–82.
17. Branch C, Cui M, Thangaswamy M, et al. Therapeutic efficacy of semisynthetic supra perfusion resuscitation fluids EAF PEG-Alb and EAF PEG Hb are differentiated by their cerebral effects in animal models of sickle cell disease. Blood. 2015;126(23):773–3.
18. Li M, Wu L, Zhao T, et al. The protective role of 5-hydroxymethyl-2-furfural (5-HMF) against acute hypobaric hypoxia. Cell Stress Chaperones. 2011;16:529–37.
19. Metcalf B, Chung C, Dufu K, et al. Discovery of GBT 440, an orally Bioavailable R-state stabilizer of sickle cell Hb. ACS Med Che Lett. 2017;8:321–6.
20. Hebbel RP, Hedlund BE. Sickle hemoglobin oxygen shifting strategies have unequal cerebrovascular risks. Am J Hematol. 2018;93:321–5.
21. Helms CC, Gladwin MT, Kim-Shapiro DB. Erythrocytes and vascular function: oxygen and nitric oxide. Front Physiol. 2018;9:125, 1–9

21

Hemoglobin-Based Blood Substitute with Pharmacological Activities of ATP, Adenosine and Reduced Glutathione: A Review of Preclinical and Early Clinical Experience

Jan Simoni

Background/Introduction

Despite decades of massive effort and hundreds of millions of dollars, the promised blood substitute is still far from fruition. There is no FDA-approved HBOC that is urgently needed to alleviate the critical global shortages of blood needed for transfusion [1–3]. The FDA agency, however, was instrumental in blood substitutes development by providing all needed scientific support and regulatory directions [4, 5]. So what went wrong? Let's mention some fundamental problems with early developments: (i) designing HBOCs without a full understanding of free Hb toxicity, (ii) inability to address and control of Hb intrinsic toxicity particularly its vasoactive and pro-oxidant/pro-inflammatory properties, (iii) lack of access to the modern biotechnological methods and tools, (iv) misaligned incentives, and (v) rushing the product to market, which led to numerous negative outcomes as focus shifts from safety to speed [1–3]. Therefore, it was not surprise that in the late phases of clinical development these products including: HemeAssist – diaspirin crosslinked human Hb (Baxter, Deerfield, IL), Hemolink – o-Raffinose polymerized human Hb (Hemosol, Inc., Mississauga, Canada), Polyheme – glutaraldehyde-polymerized pyridoxylated human Hb (Northfield Laboratories Inc., Evanston, IL), Optro, rHb1.1 – recombinant Hb expressed in Escherichia coli, (Somatogen; Eli Lilly; Baxter, Boulder, CO), Hemospan – human Hb conjugated to PEG (Sangart Inc., San Diego, CA), and PHP – pyridoxylated human Hb conjugated to PEG (Apex Bioscience, Inc., Chapel Hill, NC), have been rejected by the US FDA due to the safety and/or efficacy concerns [6, 7]. Besides HBOC-201 – glutaraldehyde-polymerized bovine Hb (Biopure Corporation; HbO2 Therapeutics LLC; OPK Biotech LLC, Cambridge, MA), which was approved by the health authorities in South Africa and Russia for treatment of anemia, the commercial development of all other products have been discontinued [8].

There is reliable experimental and clinical evidence that the tested HBOCs trigger a complex array of pathologic reactions as a result of Hb's natural feature [5, 9–17]. Physiologic clearance of plasma Hb via haptoglobin (Hp)->CD163 Hb scavenger receptor that is coupled with the heme oxygenase enzyme (HO-1) capable of transforming heme to biliverdin and carbon monoxide (CO), is required to prevent adverse effects [18–21]. This protective mechanism, however, is not effective after large rapid transfusions of HBOCs. Although chemically modified low-molecular weight Hbs can interact with CD163 via low affinity binding even in the absence of Hp, highly polymerized Hbs have no affinity to Hp and CD163, which totally impairs their physiologic clearance [18–20]. Besides, the redox-active HBOCs might participate in the synthesis of 8-isoprostanes that are known to shed CD163; thus, paradoxically blocking Hb's clearance [21]. The failure of Hb's physiologic catabolism may cause its extravasation, non-enzymatic degradation, and oxidative transformation; negatively affecting a number of vital systems, including renal, cardiovascular, gastrointestinal, neural, immunologic, coagulation, and many others [9, 17]. In circulation, HBOCs are capable of removing NO before it can interact with guanylate cyclase, reducing the synthesis of cGMP, and subsequently increasing ionized intracellular calcium to promote vasoconstriction. The extravasation of Hb and its presence in the subendothelial space and/or scavenging of NO within the vascular space are required to interfere with NO-dependent vasorelaxation [9, 16, 17, 22]. The observed changes in hemodynamics of the tested products can be related to their NO scavenging potency and ability to pass the transendothelial barrier. The transcapillary exchange of macromolecules is only minimally affected by molecular masses up to 300 kDa, but is dependent on the charge of the molecule; therefore, native Hb as well as polymerized Hb without an altered isoelectric point

J. Simoni (✉)
Texas HemoBioTherapeutics & BioInnovation Center and Texas Tech University Health Sciences Center, Lubbock, TX, USA
e-mail: jan.simoni@ttuhsc.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_21

(pI), might extravasate [9, 16, 23, 24]. HBOCs in the bloodstream autoxidize and may react with peroxides, leading to the generation of oxygen, heme, and globin based radicals, which in turn scavenge more NO while simultaneously oxidizing cellular membranes [5, 9, 13, 16, 21]. With the oxidation of arachidonic acid, 8-isoprostanes are produced, which have been implicated in Hb-mediated coronary, pulmonary and renal vasoconstriction [9, 16, 21, 25, 26]. Hb induced vasoconstriction could also be mediated by other factors. It was found that Hb activated with hydrogen peroxide, which can be present in plasma in micromolar concentrations [27], gains angiotensin converting enzyme (ACE)-like activity, being able to rapidly convert angiotensin (Ang)-I to active Ang-II; thus, becoming a contributor to the vasoconstrictive response observed immediately after HBOCs administration [28]. More recently, it was recognized that Hb itself could stimulate platelets to release serotonin (5 HT) [9, 29], which is known for the hypercontractile response of the injured arteries, particularly coronary arteries [30]. HBOCs by altering the cellular redox state may target signaling, transcriptional and translational mechanisms [9, 16, 31–33].

Hb molecule was found to be a potent inducer of nuclear factor (NF)-kappa B that is involved in the induction of many genes, including those encoding pro-inflammatory cytokines, cell adhesion molecules, selectins, tissue factor, colony stimulating factors, regulators of apoptosis, acute phase proteins, etc [31, 32]. The activation of NF-kappa B is dependent on Hb's pro-oxidant potential and the extent of Hb-mediated cellular oxidative stress that shifts the equilibrium into the oxidation state; forming a bridge between Hb-induced oxidative stress and Hb-mediated inflammatory responses [31, 32]. Therefore, redox-active HBOCs can be considered as a potent stimulator of the inflammatory cascade.

The respiratory function of Hb influences hypoxia inducible factor (HIF)-1 and HIF-2, that are responsible for induction of genes involved in erythropoiesis (erythropoietin, EPO), angiogenesis (vascular endothelial growth factor, VEGF), vascular remodeling (HO-1, nitric oxide synthase), etc. [9, 33]. Since HIF-1 and HIF-2 under normoxic conditions are hydroxylated and rapidly degradated, HBOCs with a strong hyperoxic effects (low affinity for oxygen) can participate in their degradation, unless they are stabilized via Hb-mediated oxidative phosphorylation [9, 33]. On the other hand, HBOCs with strong pressor effects or high affinity for oxygen (low P50) that promotes hypoxia, can stabilize HIF, inducing erythropoiesis [9, 33]. Assuming, that efficacious HBOCs must counteract the hypoxic and oxidant environments, the stabilization of HIF by prolonging hypoxia or oxidative phosphorylation is clinically questionable [33]. Another possible unwanted side effect of HBOCs, which has received little or no attention, is their impact on RBC-regulated vascular tone, independent from the "SNO-Hb" and "nitrite reductase activity of deoxygenated Hb" mechanisms [34]. It has been known for some time that RBC besides acting as oxygen carriers tightly control vascular tone [35]. RBCs exposed to increased shear stress, low pO_2 or acidic pH can release ATP that regulates blood flow by stimulating the P2Y-purinergic receptor that is linked to the production of vasorelaxing factors such as NO and prostacyclin (PGI_2). It was found that the proposed mechanism of the reduction of nitrite back to NO by deoxygenated Hb [34] is not responsible for the observed vasodilatory effect, but in fact is RBC->ATP-mediated [36]. It was established that nitrite enhances the release of ATP from RBCs that are the major intravascular storage of nitrites in human blood [37], via increased binding of nitrite-modified Hb to the RBC membrane that displaces glycolytic enzymes from the membrane; resulting in the formation of a pool of ATP that is released from RBC and acts as vasodilator. In other words, nitrites enhance RBC hypoxic ATP synthesis and the release of ATP into the vasculature producing P2Y->NO/PGI_2-dependent vasorelaxation. This new vasodilatory mechanism does not require the release of NO from the RBCs. Although a few

details need to be ironed out, it is obvious that HBOCs may affect the vasodilatory function of RBCs with or without the presence of nitrites. It seems that the products with a high affinity for oxygen (low P50) and elevated viscosity (high shear stress) may stimulate RBCs to release vasodilatory ATP [38]. However, high-affinity products with P50 of only 5–6 mmHg may impair optimum oxygen delivery. On the other hand, HBOCs with a low affinity for oxygen (i.e., 38–40 mm Hg) may over-saturate the blood; thus blocking the release of ATP from RBCs, causing vasoconstriction. The blockage of ATP release by these products can be potentiated by decreased viscosity that lowers the shear stress. The present knowledge on Hb intrinsic toxicity could assure successful development of an effective HBOC. It is important to have all the documented intrinsic Hb toxic mechanisms under control, particularly the oxidative, inflammatory and vasoconstrictive pathways, before considering any HBOC as safe and efficacious.

Main Body of the Chapter

Realization Principle of HemoTech Pharmacologic Cross-linking

In order to eradicate Hb intrinsic toxicity, we have created the concept of "pharmacologic cross-linking" [39, 40]. This chemical modification technology does not interfere with Hb respiratory function, but provides Hb molecules with new medicinal properties. It addresses problems such as vasoconstriction, oxidative stress, and inflammation. The resulting

product, HemoTech, is a pure bovine Hb intramolecularly cross-linked with open ring (o)-ATP and intermolecularly with open ring (o)-adenosine, and decorated with reduced glutathione (GSH). This chemical modification procedure in addition to stabilizing polymeric structure provides the desired pharmacologic activities of ATP, adenosine and GSH (Fig. 21.1). The Hb cross-linking reagents besides possessing the desired pharmacologic activities are classified as "affinity directed" beta-beta cross-linkers. The chemical modification done with these cross-linkers can produce a "steering effect" and acceleration of the preferential reaction at the beta-beta interface [39, 40]. In this product, the reaction with o-ATP stabilizes the Hb tetramer and prevents its dimerization, and the reaction with o-adenosine allows the creation of Hb-oligomers, avoiding the formation of toxic high molecular weight polymers (Fig. 21.1).

In the HemoTech polymeric structure, ATP besides tetramer stabilization produces the vasodilatory effect through stimulation of the P2Y receptors that are linked to NO and PGI_2 production (Fig. 21.2a). This action of ATP on P2Y is autonomous and not linked with the induction of ATP release from RBCs by HBOCs with high oxygen affinity and viscosity [38]. Therefore, ATP in HemoTech is an

independent regulator of vascular tone, not affected by P50, pH or shear stress/viscosity (Fig. 21.2a). The adenosine is used to counteract the vasoconstrictive and pro-inflammatory properties of Hb with the activation of adenosine $A_{2A\&B}$ receptors, which produce vasodilatation, moderation of inflammatory reactions and prevention of platelet aggregation. While the activation of adenosine A_3 receptor provides cytoprotection, induction of adenosine A_1 receptor results in neuroprotection (Fig. 21.2b). The conjugation of Hb with an anionic peptide – GSH shields heme from the reactive oxygen species (ROS), thus lowering Hb's pro-oxidant potential and by blocking NO access and forming S-Nitrosoglutathione (SNO) attenuates Hb's vasoconstrictive potential. At the same time, GSH that has antioxidant properties introduces more electronegative charge onto the surface of the Hb molecule that blocks Hb's transglomerular and transendothelial passage, and make it less visible to phagocytes (Fig. 21.2c). The alteration of surface charge of HemoTech polymers and tetramers to the same extent is one of the essential features of this novel modification procedure. The electrophoretic mobility study demonstrated that all Hb polymers have a uniform electronegative surface charge with a pI of 6.1–6.3. This new electrostatic property of Hb molecules has blocked their transglomerular and transendothelial passage, thus attenuating endothelial pathological responses and nephrotoxic reactions [16, 31, 32, 41–44].

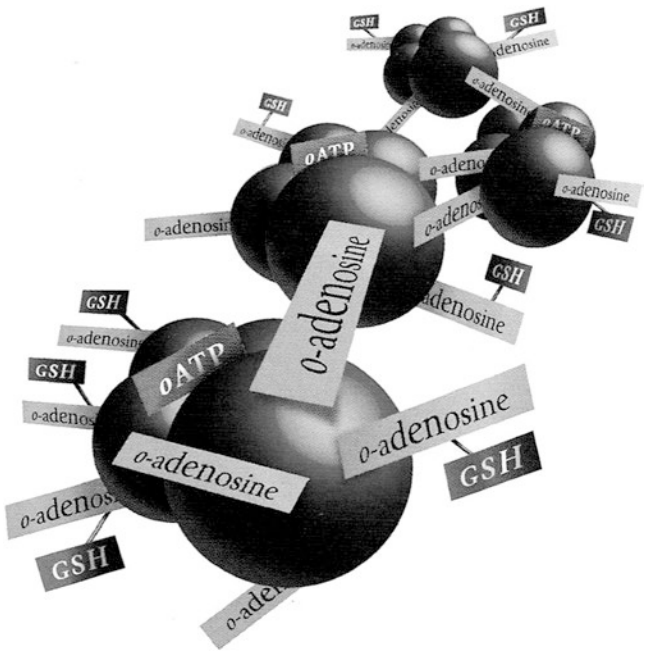


Fig. 21.1 Graphic Representation of the HemoTech Molecule. HemoTech is composed of pure bovine Hb, cross-linked intramolecularly with o-ATP and intermolecularly with o-adenosine, and conjugated with reduced glutathione (GSH)

HemoTech Production Under the Concurent Good Manufacturing Practice (cGMP) Regulations

HemoTech manufacturing includes a novel, validated orthogonal technology platform for an effective clearance of prions that cause bovine spongiform encephalopathy (BSE) and Creutzfels-Jakob disease (CJD)) and non-enveloped and enveloped viruses: BVDV, BCoV, XMuLv, EMCV, IRB and BPV [45, 46]. The clearance validation tests performed in compliance with the Good Laboratory Practice (GLP) standards at BioReliance/Invitrogen Corp. (Rockville, MD), confirmed that HemoTech production technology is extremely effective in the elimination of prions and viruses. While the clearance of enveloped and non-enveloped viruses was in average 1–4 additive log reduction values (LRV's) above the FDA limits, the prion elimination was more than 10 LRV's, exceeding by 5 LRV's the FDA requirements [44–46]. HemoTech manufacturing technology also includes the elimination of endotoxin and steps to ensure product sterility [39, 40]. HemoTech is formulated as a 6.5 g/dL solution, enriched with electrolytes and mannitol. The final formulation is isotonic (300–325 mOsm/L) and is free of bacterial, viral and prion pathogens and endotoxin. This product does not contain polymers above 500 kDa and contains a trace of tetramers and less that 5% of met-Hb. HemoTech has a uniformed electronegative charge, and P50 that maintains a proper oxygen delivery index [39, 40]. HemoTech could be formulated in oxy-or CO-form. The typical physico-chemical characteristics of this product are listed below:

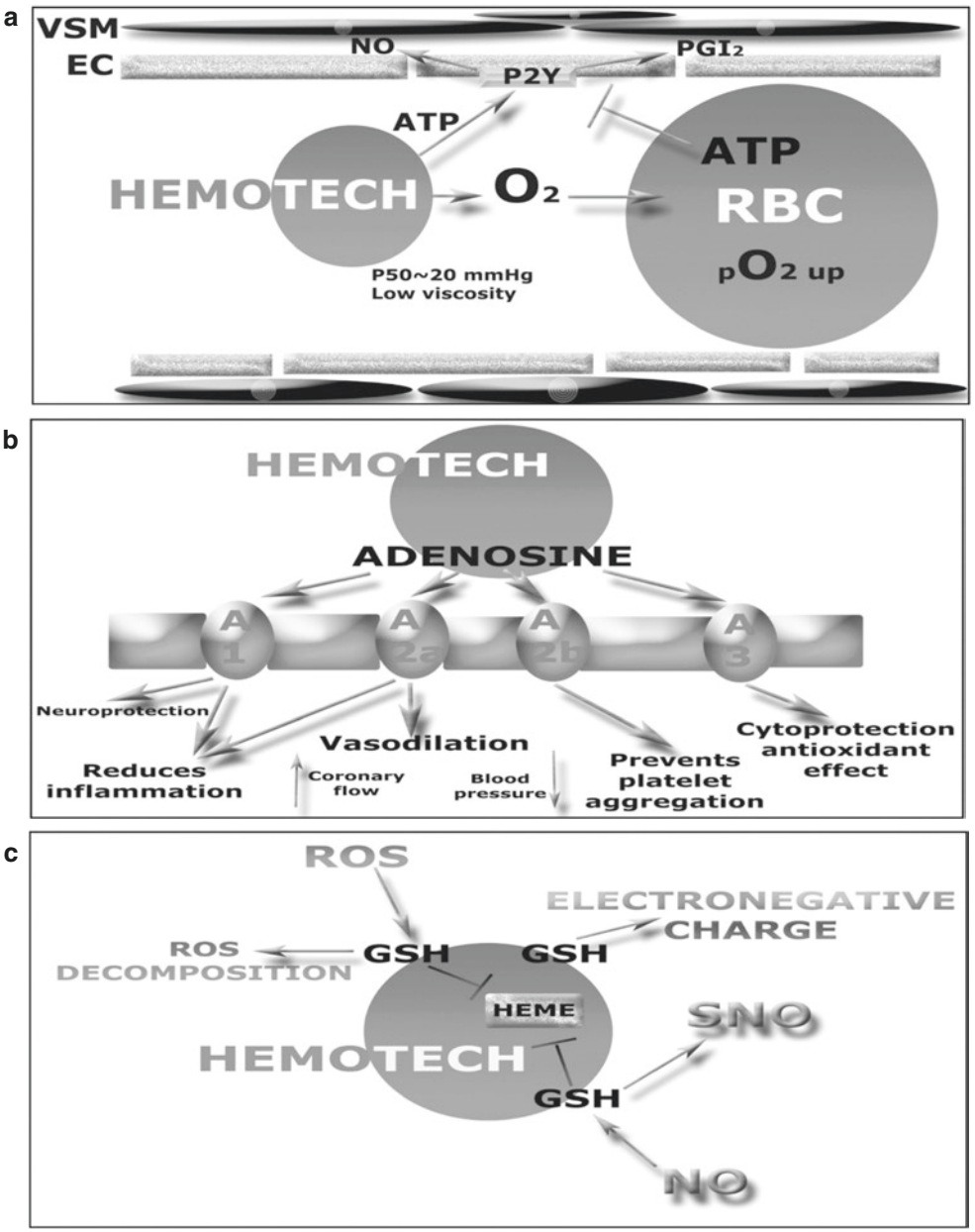


Fig. 21.2 HemoTech Physiological and Pharmacological Effects. (**a**) Schematic Diagram of ATP's Role in HemoTech. ATP stabilizes Hb tetramers and produces the vasodilatory effect via stimulation of the P2Y receptors linked to NO and PGI2 synthesis. (**b**) Graphic Representation of Adenosine's Role in HemoTech. Adenosine stimulates A1 receptors involved in neuroprotection, A2 receptors responsible for vasodilation, moderation of inflammatory reactions and inhibition of platelet aggregation, and A3 receptors that provide cytoprotection. (**c**) Schematic Diagram of Reduced Glutathione (GSH) role in HemoTech. GSH introduces electronegative charges onto HemoTech surface (pI 6.1–6.3), shields heme from the reactive oxygen species (ROS) thus lowering Hb's pro-oxidant potential, and by blocking NO access to heme and forming S-Nitrosoglutathione (SNO) attenuates Hb's vasoconstrictive potential

- Hb (g/dL): 6.5
- Oxy-Hb (%): >95
- Met-Hb % of oxy-Hb: <5
- CO-Hb % of oxy-Hb: <5 (HemoTech in CO-form: CO-Hb%: >95)
- pH U: 7.8–8.1 (THAM 20 mM)
- Sodium: 140 mmol/L
- Potassium: 4 mmol/L
- Chloride: 100 mmol/L
- Sodium Lactate: 27 mmol/L
- Calcium: 1.3 mmol/L
- Mannitol: 0.8 mg/mL
- Colloid Oncotic Pressure mmHg: 20–23
- Osmolarity: mOsm/kg: 300–325 (after adding of electrolytes and mannitol)
- Oxygen Affinity (P50) mmHg: 20–26 (dependent on Cl concentration)
- Polymeric profile: tetramers: <5%, polymers: <500,000 daltons 95%
- Isoelectric Point (pI): 6.1–6.3

As a part the Chemistry Manufacturing and Controls Information (CMC), HemoTech was subjected for stability testing at different temperatures (-90 °C, -20 °C, +4 °C, +22 °C, +37 °C and +42 °C) and time intervals (ranging from days to years). The stability tests performed on HemoTech

included: (i) heme oxidation, (ii) polymeric structure integrity, (iii) surface charge changes. The tests showed that HemoTech stored in its reduced, oxy-form resists oxidation at -90 °C up to 5 years; at +4 °C up to 6 months; at +37 °C up to 2 days; and at +42 °C up to 1 day; and did not change with respect to charge and polymer composition when stored at -90 °C up to 5 years; at +4 °C up to 12 months; at +37 °C and +42 °C up to 3 days. HemoTech in CO-form at -90 °C can be stores up to 10 years and up to 1 year at +4 °C.

HemoTech Preclinical Studies

The regulatory mandated requirements have been met including CMC and non-clinical pharmacology, toxicology, genotoxicity and efficacy studies.

The pharmacology models (Pharm) used to evaluate HemoTech included: Pharm 1: Hemodynamics and tissue oxygen delivery in a hemorrhagic shock model [47], Pharm 2: Ability to raise cAMP, prostacyclin (PGI_2) (stimulation of the adenosine and purinergic P2Y receptors) and its reactivity with endothelial NO [16,31,32,41,44, In-house report], Pharm 3: Effects on human blood components [48], Pharm 4: Anti-inflammatory, anti-oxidant and anti-apoptotic activities toward human endothelial cells [16, 31, 32, 44, 48, 49], Pharm 5: Effects on human brain astrocytes and neurons [43], Pharm 6: Reactivity with hydrogen peroxide and its ability to participate in the synthesis of vasoconstrictive 8-isoprostane and endothelin-1 [13, 16, 21, 26, 31, 41, 48, 50], and Pharm 7: Transcriptional, translational and erythropoietic potential [16, 31–33, 41, 44, 51].

The pharmacokinetics models (PK) included: PK 1: Determination of PK/PD in Coebus monkeys [in-house report], PK 2: Determination of circulatory half-life and renal clearance in Sprague-Dawley rats [42], PK 3: Determination of circulatory half-life and renal and hepatic clearance in New Zealand rabbits [in-house report], PK 4: Human monocyte/macrophage clearance [15, 48, 51], and PK 5: Determination of endothelial clearance and permeability in human coronary artery and brain microvascular endothelial cells [16, 32, 41, 49].

The toxicological profile of HemoTech has been determined in: (i) acute toxicity studies in Sprague-Dawley rats, domestic crossbred pigs, New Zealand rabbits, Capuchin monkeys, Beagle dogs, (ii) 7-day repeat dose studies in Sprague-Dawley rats and Beagle dogs, (iii) Guinea pig multi dose and dermal sensitization study, and (v) standard battery of genotoxicity assays: (a) reverse mutation in Salmonella typhimurium, (b) chromosome aberration in human lymphocytes in vitro, (c) gene mutation in Chinese hamster V79 cells, (d) unscheduled DNA synthesis in primary rat hepatocytes,(e) in vitro mammalian bone marrow cytogenetic test, and (f) immunogenicity studies.

These preclinical tests were conducted in-house and at the RTC Research Toxicology Centre S.p.A. (Pompesia, Rome, Italy) in accordance with GLP regulations of the US FDA (21 CFR Part 58) and the principles of GLP relating to the conduct of non-clinical laboratory studies and other regulations [52]. In addition effects of HemoTech on appropriate physiological measures in human cell systems (coronary artery endothelial cells, brain capillary endothelial cells, lung microvascular endothelial cells, monocytes, macrophages, astrocytes, neurons, RBC, platelets) normal animals and disease models have been also determined [44].

HemoTech was found to reduce the peripheral vascular resistance, an action mediated by the adenosine A_2 receptor. HemoTech also produced vasodilation by activation of a P2Y receptor with ATP, which serves as a intramolecular cross-linker. The vasodilatory effect was accelerated by reaction of the endothelial NO with the molecules of GSH, which are chemically attached to the HemoTech surface, presumably producing S-nitroso-GSH. This conclusion is based on indirect evidence provided by the following two observations, one associated with lower oxidation rate of NO in the presence of HemoTech as compared to unmodified Hb, the other with very low oxidation rate of heme in the presence of NO. Therefore, it can be concluded that the GSH shield besides preserving NO also prevented the NO-induced oxidation of heme. HemoTech did not appear to aggravate cellular oxidative stress, or to activate inflammatory responses. Selective targeting amino acid residues of Hb beta chains with adenosine, and incorporation of GSH achieved reduction of the natural pro-oxidative potential of Hb . HemoTech when reacted with hydrogen peroxide did not oxidatively modify LDLs or cross-link apo B. It seems that "steering effect" and acceleration of the preferential reaction at the beta-beta interface makes tyrosine unavailable for reaction with hydrogen peroxide, thus preventing the formation of heme globin radicals. This chemical/pharmacological modification procedure also significantly decreased the formation of heme ferryl iron.

HemoTech did not change the cellular redox equilibrium nor did it activate apoptotic responses. This product was found to eliminate NF-kappa B-induction. Even in GSH depleted cells; this blood substitute did not activate the inflammatory responses. Another element that contributed to its anti-inflammatory action was low endothelial calcium flux and low formation of 8-isoprostanes, well known activators of protein kinases that destabilize NF-kappa B. HemoTech also stimulates the adenosine A_3 receptor, responsible for cytoprotection]. It was demonstrated that stimulation of this receptor activates the cellular anti-oxidant enzyme system, thus stabilizing the redox state. In the study, using GSH depleted human brain capillary and coronary artery endothelial cells, HemoTech prevented NF-kappa B induction and facilitated HIF-1/2 stabilization and DNA binding to the EPO gene under normoxia. HemoTech possesses a unique erythropoietic potential. Using an in vitro

cellular model, we investigated the molecular mechanisms of erythropoietic action of HemoTech.

While unmodified tetrameric Hb suppresses erythropoiesis by increasing the cytoplasmic degradation of hypoxia HIF-1/2 and decreasing binding to the EPO gene while inducing NF-kappa B-dependent anti-erythropoietic genes, HemoTech accelerates erythropoiesis by downregulating NF-kappa B, stabilizing and facilitating HIF-1/2 binding to the EPO gene, under both oxygen conditions. ATP and adenosine contribute to normoxic stabilization of HIF-1/2 and, with GSH, inhibit the NF-kappa B pathway that is involved in the suppression of erythroid-specific genes. The erythropoietic effect of HemoTech was observed in rats, rabbits, monkeys and humans [33, 40, 52].

The toxicological profile of HemoTech, which has been determined in acute and 7-day repeat dose studies in rats and dogs, in guinea pig dermal sensitivity assays and in the standard battery of genotoxicity assays, as well as in appropriate physiological measures in cell systems, normal animals and disease models, demonstrates that this product is non-toxic and effective under these experimental conditions [52]. In acute studies in rats, pigs, monkeys and dogs, HemoTech was shown to be free of toxicity. There were no notable differences between the control and treated animals throughout the 7-day observation period or at necropsy. The 7-day repeat dose studies performed in rats and dogs, daily treatment with up to 1,300 mg/kg/day did not result in any significant toxicity [52]. Multi-dose Guinea pig dermal sensitization studies and tests for foreign proteins indicated that HemoTech is not antigenic in this species [52]. HemoTech did not induce increases in chromosomal aberrations compared to controls, either in the presence or absence of S9 activation [52]. Also this product did not induce gene mutations in Chinese hamster V79 cells either in the presence or absence of S9 activation [52]. In vitro unscheduled DNA synthesis (UDS) in primary rat hepatocytes and in vivo mouse micronucleus tests provided further evidence that HemoTech lacks genotoxicity [52].

The HemoTech circulatory half-life is approximately 24 hours. While in the circulation, the structure of HemoTech is unchanged for up to 3 hours. After that HemoTech's polymers with molecular mass similar to Hb-Hp complex are slowly removed from the circulation. At the 24 hour interval, modified tetramers, octamers, decamers and larger molecular structures are still present in the circulation. Although HemoTech-Hp binding has not been investigated, a significant increase in total bilirubin level and the lack of HemoTech renal excretion, suggest its hepatic clearance. The spectral analysis at the 24 hour interval showed that HemoTech remained in its reduced form.

The results of these pre-clinical studies are favorable, indicating that HemoTech has vasodilatory activity and can reduce vasoconstriction that follows hemorrhage, has erythropoietic activity, and produces no adverse nephrotoxic, neurotoxic, oxidative and inflammatory reactions.

HemoTech Clinical Studies

HemoTech's clinical proof-of-concept was performed at the Centre se l'Anemie S. S. (Kinshasa, Zaire) by the Instituto Sierovaccinogeno Italiano – ISI (S. Antimo-Napoli, Italy) after obtaining approval by the Ethics Committee of the Institut de la Recherche en Sciences de la Sante' (Kinshasa, DRC). HemoTech's was clinically investigated in children with sickle cell anemia who suffered from an "aplastic crisis" manifested by a sudden decrease in Hb concentration associated with absence of reticulocytes and "vaso-occlusive episodes." After infusion of HemoTech in a volume corresponding to 25% of TBV no adverse reactions were noted. To the contrary, in children with aplastic crisis, HemoTech stimulated the bone marrow to a significant erythropoietic effect, whereby the number of reticulocytes increased from zero to 47±7%. In children with vaso-occlusive crises, pain was quickly relieved. HemoTech showed beneficial therapeutic effects in all patients. In fact, HemoTech did not only alleviate the inflammatory reactions in sickle cell anemia patients, but also produced an effective erythropoietic response [53].

More recently HemoTech was tested ex vivo on human platelets obtained from percutaneous coronary intervention (PCI) patients. It was found that HemoTech decreased platelet aggregability in response to platelet aggregation agonists, particularly collagen, and blocked the release of serotonin (5-HT) [54]. Based on this observation and previous studies that proved the ability of HemoTech to optimize oxygen delivery and induce vasodilation, alleviation of oxidative reactions and reduction of the endothelial inflammatory responses, this HBOC is considered to be therapeutically useful as a perfusion fluid for PCI procedures. It is believed that HemoTech may have the potential to mitigate myocardial ischemia and thrombotic events associated with PCI. Other therapeutic applications for HemoTech are under investigation [55].

Summary

The results of preclinical and clinical studies indicate that HemoTech, a bovine Hb cross-linked intramolecularly with ATP and intermolecularly with adenosine, and conjugated with GSH, has the following properties: (i) can work as a physiological oxygen carrier and produces no adverse nephrotoxic, neurotoxic, oxidative, or inflammatory reactions, (ii) vasodilatory activity and can reduce the vasoconstriction that follows hemorrhage, (iii) prolong intravascular persistence and can sustain plasma volume, and (iv) erythropoietic activities. It seems that pharmacologic cross-linking of Hb with ATP, adenosine and GSH provides the desired pharmacologic activities of counteracting vasoconstrictive, pro-oxidative and pro-inflammatory properties of Hb. Based on the preclinical and clinical testing, it can be concluded that this novel pharmacological modification method has allowed the prepara-

tion of a non-toxic and efficacious solution, which promises to be an effective HBOC for various clinical indications.

Key Points

- A novel concept of "pharmacologic cross-linking" of the Hb molecule with ATP, adenosine and GSH proved to be feasible and useful for designing an effective blood substitute, HemoTech.
- The "pharmacologic cross-linking" does not interfere with Hb respiratory function, but eradicates Hb's intrinsic toxicity and provides Hb molecule with new medicinal properties of ATP, adenosine and GSH.
- The results of preclinical and clinical studies indicate that HemoTech in non-toxic and works as physiologic oxygen carrier with prolonged intravascular persistence.
- HemoTech is vasodilatory and can reduce vasoconstriction that follows hemorrhage, has antioxidant and anti-inflammatory activities, and erythropoietic properties.
- HemoTech promises to be an effective blood substitute for various clinical indications.

References

1. Simoni J. Artificial oxygen carriers: scientific and biotechnological points of view. Artif Organs. 2009;33(2):92–6. https://doi.org/10.1111/j.1525-1594.2008.00691.x.
2. Simoni J. Artificial oxygen carriers: renewed commercial interest and scientific/technological advances. Artif Organs. 2012;36(2):123–6. https://doi.org/10.1111/j.1525-1594.2011.01430.x.
3. Simoni J. New approaches in commercial development of artificial oxygen carriers. Artif Organs. 2014;38(8):621–4. https://doi.org/10.1111/aor.12371. Epub 2014 Aug 12
4. Guidance for Industry. Criteria for safety and efficacy evaluation of oxygen therapeutics as red cell substitutes. Rockville: U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research; 2004. p. 1–19.
5. Alayash AI. Hemoglobin-based blood substitutes: oxygen carriers, pressor agents, or oxidants? Nat Biotechnol. 1999;17(6):545–9. https://doi.org/10.1038/9849.
6. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA. 2008;299(19):2304–12. https://doi.org/10.1001/jama.299.19.jrv80007. Epub 2008 Apr 28. Erratum in: JAMA. 2008 Sep 17;300(11): 1300.
7. Silverman TA, Weiskopf RB, Planning Committee and the Speakers. Hemoglobin-based oxygen carriers: current status and future directions. Anesthesiology. 2009;111(5):946–63. https://doi.org/10.1097/ALN.0b013e3181ba3c2c.
8. Jahr JS, Moallempour M, Lim JC. HBOC-201, hemoglobin glutamer-250 (bovine), Hemopure (Biopure Corporation). Expert Opin Biol Ther. 2008;8(9):1425–33. https://doi.org/10.1517/14712598.8.9.1425.
9. Simoni J, Simoni G, Moeller JF. Intrinsic toxicity of hemoglobin: how to counteract it. Artif Organs. 2009;33(2):100–9. https://doi.org/10.1111/j.1525-1594.2008.00693.x.
10. Feola M, Simoni J, Dobke M, Canizaro PC. Complement activation and the toxicity of stroma-free hemoglobin solutions in primates. Circ Shock. 1988;25(4):275–90.
11. Feola M, Simoni J, Tran R, Canizaro PC. Mechanisms of toxicity of hemoglobin solutions. Biomater Artif Cells Artif Organs. 1988;16(1–3):217–26. https://doi.org/10.3109/10731198809132571.
12. Feola M, Simoni J, Canizaro PC, Tran R, Raschbaum G, Behal FJ. Toxicity of polymerized hemoglobin solutions. Surg Gynecol Obstet. 1988;166(3):211–22.
13. Simoni J, Feola M, Canizaro PC. Generation of free oxygen radicals and the toxicity of hemoglobin solutions. Biomater Artif Cells Artif Organs. 1990;18(2):189–202. https://doi.org/10.3109/10731199009117301.
14. Feola M, Simoni J, Tran R, Canizaro PC. Nephrotoxicity of hemoglobin solutions. Biomater Artif Cells Artif Organs. 1990;18(2):233–49. https://doi.org/10.3109/10731199009117304.
15. Simoni J, Feola M, Tran R, Buckner M, Canizaro PC. Biocompatibility of hemoglobin solutions. II. The inflammatory reaction of human monocytes and mouse peritoneal macrophages. Artif Organs. 1990;14(2):98–109. https://doi.org/10.1111/j.1525-1594.1990.tb01603.x.
16. Simoni J. Endothelial cell response to hemoglobin based oxygen carriers. Is the attenuation of pathological reactions possible? In: Kobayashi K, Tsuchida E, Horinouchi H, editors. Artificial oxygen carrier. Its front line. Tokyo-Berlin-Heidelberg-New York: Springer-Verlag; 2005. p. 75–125.
17. Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. JAMA. 2005;293(13):1653–62. https://doi.org/10.1001/jama.293.13.1653.
18. Schaer DJ, Schaer CA, Buehler PW, Boykins RA, Schoedon G, Alayash AI, Schaffner A. CD163 is the macrophage scavenger receptor for native and chemically modified hemoglobins in the absence of haptoglobin. Blood. 2006;107(1):373–80. https://doi.org/10.1182/blood-2005-03-1014. Epub 2005 Sep 27
19. Schaer DJ, Buehler PW, Alayash AI, Belcher JD, Vercellotti GM. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. Blood. 2013;121(8):1276–84. https://doi.org/10.1182/blood-2012-11-451229. Epub 2012 Dec 20. PMID: 23264591; PMCID: PMC3578950.
20. Buehler PW, Humar R, Schaer DJ. Haptoglobin therapeutics and compartmentalization of cell-free hemoglobin toxicity. Trends Mol Med. 2020;26(7):683–97. https://doi.org/10.1016/j.molmed.2020.02.004. Epub 2020 Mar 21
21. Simoni J, Simoni G, Griswold JA, Moeller JF, Tsikouris JP, Khanna A, Roongsritong C, Wesson DE. Role of free hemoglobin in 8-iso prostaglandin F2-alpha synthesis in chronic renal failure and its impact on CD163-Hb scavenger receptor and on coronary artery endothelium. ASAIO J. 2006;52(6):652–61. https://doi.org/10.1097/01.mat.0000235282.89757.9f.
22. Sampei K, Ulatowski JA, Asano Y, Kwansa H, Bucci E, Koehler RC. Role of nitric oxide scavenging in vascular response to cell-free hemoglobin transfusion. Am J Physiol Heart Circ Physiol. 2005;289(3):H1191–201. https://doi.org/10.1152/ajpheart.00251.2005. Epub 2005 May 13. PMID: 15894576; PMCID: PMC1819403.
23. Nakai K, Sakuma I, Ohta T, Ando J, Kitabatake A, Nakazato Y, Takahashi TA. Permeability characteristics of hemoglobin derivatives across cultured endothelial cell monolayers. J Lab Clin Med. 1998;132(4):313–9. https://doi.org/10.1016/s0022-2143(98)90045-2.
24. Baldwin AL. Modified hemoglobins produce venular interendothelial gaps and albumin leakage in the rat mesentery. Am J Phys. 1999;277(2):H650–9. https://doi.org/10.1152/ajpheart.1999.277.2.H650.
25. Fonseca V, Avizinis J, Moon-Massat P, Freilich D, Kim HW, Hai CM. Differential sensitivities of pulmonary and coronary arteries to hemoglobin-based oxygen carriers and nitrovasodilators: study in a bovine ex vivo model of vascular strips. Vascul

Pharmacol. 2010;52(5–6):215–23. https://doi.org/10.1016/j.vph.2009.12.005. Epub 2009 Dec 22. PMID: 20026426; PMCID: PMC2859108.
26. Simoni J, Villanueva-Meyer J, Simoni G, Moeller JF, Wesson DE. Control of oxidative reactions of hemoglobin in the design of blood substitutes: role of the ascorbate-glutathione antioxidant system. Artif Organs. 2009;33(2):115–26. https://doi.org/10.1111/j.1525-1594.2008.00695.x.
27. Halliwell B, Clement MV, Long LH. Hydrogen peroxide in the human body. FEBS Lett. 2000;486(1):10–3. https://doi.org/10.1016/s0014-5793(00)02197-9.
28. Simoni J, Simoni G, Moeller JF, Tsikouris JP, Wesson DE. Evaluation of angiotensin converting enzyme (ACE)-like activity of acellular hemoglobin. Artif Cells Blood Substit Immobil Biotechnol. 2007;35(2):191–210. https://doi.org/10.1080/10731190601188273. PMID: 17453704.
29. Simoni J, Simoni G, Roongsritong C, Moeller JF, Simoni P, Griswold JA, Wesson DE. Novel contributing factor to intrinsic toxicity of hemoglobin: serotonin. The XIth international symposium on blood substitutes, Beijing, China, October 18–21, 2007. Abstract Book 2007;107(PS-10).
30. Van Nueten JM, Janssens WJ, Vanhoutte PM. Serotonin and vascular reactivity. Pharmacol Res Commun. 1985;17(7):585–608. https://doi.org/10.1016/0031-6989(85)90067-0.
31. Simoni J, Simoni G, Lox CD, Prien SD, Shires GT. Modified hemoglobin solution, with desired pharmacological properties, does not activate nuclear transcription factor NF-kappa B in human vascular endothelial cells. Artif Cells Blood Substit Immobil Biotechnol. 1997;25(1–2):193–210. https://doi.org/10.3109/10731199709118910.
32. Simoni J, Simoni G, Lox CD, Prien SD, Tran R, Shires GT. Expression of adhesion molecules and von Willebrand factor in human coronary artery endothelial cells incubated with differently modified hemoglobin solutions. Artif Cells Blood Substit Immobil Biotechnol. 1997;25(1–2):211–25. https://doi.org/10.3109/10731199709118911.
33. Simoni J, Simoni G, Moeller JF, Feola M, Griswold JA, Wesson DE. Adenosine-5′-triphosphate-adenosine-glutathione cross-linked hemoglobin as erythropoiesis-stimulating agent. Artif Organs. 2012;36(2):139–50. https://doi.org/10.1111/j.1525-1594.2011.01431.x.
34. Kim-Shapiro DB, Gladwin MT, Patel RP, Hogg N. The reaction between nitrite and hemoglobin: the role of nitrite in hemoglobin-mediated hypoxic vasodilation. J Inorg Biochem. 2005;99(1):237–46. https://doi.org/10.1016/j.jinorgbio.2004.10.034.
35. Ellsworth ML, Forrester T, Ellis CG, Dietrich HH. The erythrocyte as a regulator of vascular tone. Am J Phys. 1995;269(6 Pt 2):H2155–61. https://doi.org/10.1152/ajpheart.1995.269.6.H2155.
36. Cao Z, Bell JB, Mohanty JG, Nagababu E, Rifkind JM. Nitrite enhances RBC hypoxic ATP synthesis and the release of ATP into the vasculature: a new mechanism for nitrite-induced vasodilation. Am J Physiol Heart Circ Physiol. 2009;297(4):H1494–503. https://doi.org/10.1152/ajpheart.01233.2008. Epub 2009 Aug 21. PMID: 19700624; PMCID: PMC3215427.
37. Dejam A, Hunter CJ, Pelletier MM, Hsu LL, Machado RF, Shiva S, Power GG, Kelm M, Gladwin MT, Schechter AN. Erythrocytes are the major intravascular storage sites of nitrite in human blood. Blood. 2005;106(2):734–9. https://doi.org/10.1182/blood-2005-02-0567. Epub 2005 Mar 17. PMID: 15774613; PMCID: PMC1895176.
38. Cole RH, Malavalli A, Vandegriff KD. Erythrocytic ATP release in the presence of modified cell-free hemoglobin. Biophys Chem. 2009;144(3):119–22. https://doi.org/10.1016/j.bpc.2009.07.004. Epub 2009 Aug 3
39. Feola M, Simoni J, Canizaro PC: Blood substitute. U.S. Patent No. 5,439,882.
40. Simoni J, Simoni G, Feola M: Methods of treating acute blood loss. U.S. Patent No. 7,759,306.
41. Simoni J, Simoni G, Martinez-Zaguilan R, Wesson DE, Lox CD, Prien SD, Kumar RV. Improved blood substitute: evaluation of its effects on human endothelial cells. ASAIO J. 1998;44(5):M356–67.
42. Simoni J, Simoni G, Hartsell A, Feola M. An improved blood substitute. In vivo evaluation of its renal effects. ASAIO J. 1997;43(5):M714–25.
43. Simoni J, Simoni G, Wesson DE, Griswold JA, Moeller JF, Feola M. Combining hemoglobin with adenosine and reduced glutathione attenuates its direct and direct neurotoxic potential. 9th international symposium on blood substitutes, Tokyo, Japan, March 2–5, 2003. Volume: Artif Blood. 2003;11(1):96.
44. Simoni J, Simoni G, Wesson DE, Feola M. ATP-adenosine-glutathione cross-linked hemoglobin as clinically useful oxygen carrier. Curr Drug Discov Technol. 2012;9(3):173–87. https://doi.org/10.2174/157016312802650797.
45. Simoni J, Simoni G, Moeller JF. Orthogonal method for the removal of transmissible spongiform encephalopathy agents from biological fluids. Patent Application US20110097746. Publication Date: 04/28/2011.
46. Simoni J, Simoni G, Moeller JF. Orthogonal method for the removal of prions and viruses from hemoglobin solution. The XIII international symposium on blood substitutes and oxygen therapeutics. Massachusetts General Hospital and Harvard Medical School, Boston, MA, July 27–29, 2011. Abstracts Book 2011;69(P-28).
47. Simoni J, Simoni G, Newman G, Feola M. An improved blood substitute. In vivo evaluation of its hemodynamic effects. ASAIO J. 1996;42(5):M773–82.
48. Simoni J, Simoni G, Wesson DE, Griswold JA, Feola M. A novel hemoglobin-adenosine-glutathione based blood substitute: evaluation of its effects on human blood ex vivo. ASAIO J. 2000;46(6):679–92. https://doi.org/10.1097/00002480-200011000-00007.
49. Simoni J, Simoni G, Wesson DE, Griswold JA, Feola M. The response of human brain capillary endothelial cells to a novel hemoglobin-adenosine-glutathione based red cell substitute. The 5th international conference on current issues in blood substitute research. Stockholm, Sweden, June 6–8, 2002. Volume: Artif Cells Blood Substit Biotechnol 2002;31:486–487.
50. Simoni J, Simoni G, Wesson DE, Griswold JA, Feola M. Attenuation of hemoglobin pro-oxidant potential by cross-linking with adenosine and conjugation with reduced glutathione. The 47th annual conference of the American Society for artificial internal organs, New York, NY, June 7–9, 2001. Volume: ASAIO J. 2001;47(2)172.
51. Simoni J, Simoni G, Lox CD, McGunegle DE, Feola M. Cytokines and PAF release from human monocytes and macrophages: effect of hemoglobin and contaminants. Artif Cells Blood Substit Immobil Biotechnol. 1994;22(3):525–34. https://doi.org/10.3109/10731199409117880.
52. HemoTech GLP. Preclinical toxicological tests. Final reports. Pompesia: RTC Research Toxicology Centre S.p.A.
53. Feola M, Simoni J, Angelillo R, Luhruma Z, Kabakele M, Manzombi M, Kaluila M. Clinical trial of a hemoglobin based blood substitute in patients with sickle cell anemia. Surg Gynecol Obstet. 1992;174(5):379–86.
54. Simoni J, Simoni G, Moeller JF, Kumar A, Roongsritong C, Meyerrose GE. HemoTech-blood substitute rle in the preventing of platelet activation in percutaneous coronary intervention (PCI) patients. The 56th ASAIO annual conference, Baltimore, MD, May 27–29, 2010. Volume: ASAIO J. 2010;56(2):108.
55. Simoni J, Simoni G, Moeller JF, Feola M, Wesson DE. Artificial oxygen carrier with pharmacologic actions of adenosine-5′-triphosphate, adenosine, and reduced glutathione formulated to treat an array of medical conditions. Artif Organs. 2014;38(8):684–90. https://doi.org/10.1111/aor.12337. Epub 2014 Jul 1

Potential Clinical Application of Hemoglobin Vesicles as an Artificial Oxygen Carrier and Carbon Monoxide Carrier

22

Hiromi Sakai, Naoko Kobayashi, Tomoko Kure, and Hiroshi Azuma

Introduction

Blood donation and transfusion systems have contributed considerably to human healthcare. However, even in these modern days, blood is unavailable for patients in some situations, for example, in a prehospital situation after a traffic accident or obstetric hemorrhage, in remote rural areas, after natural disasters, and after terrorist attacks. It is therefore necessary to consider blood supply systems for such emergency situations. Moreover, the progressing aging society and the present COVID-19 epidemic strongly influence the collection of sufficient numbers of blood donors [1]. To resolve such blood-related problems, research and development of artificial red cells (hemoglobin vesicles, Hb-V) is going on in Japan, aimed at the realization of a transfusion alternative for clinical use [2]. Outdated donated RBCs can be regenerated as storable artificial red cells that are free from contamination by pathogens and blood type antigens. Immediate injection of Hb-V on site is expected to be able to save lives when blood is unavailable. Our academic consortium has clarified the safety and efficacy of HbV as a transfusion alternative. Moreover, some benefits of HbV compared to RBCs such as small size, stability, and handling suggest other potential clinical applications such as organ preservation fluid, photosensitizer, and CO carriers [3].

Why is encapsulation of Hb necessary? Actually, although Hb is the most abundant protein in blood, Hb is compartmentalized in RBCs with intracellular concentration of about 35 g/dL. In spite of such abundance in blood as a binding site of oxygen, Hb induces various toxicities once it is released from RBCs during blood circulation, such as dissociation into dimers for extravasation [4], renal and neurological toxicities, and vasoconstriction because of high reactivity of Hb with NO as endothelium derived vasorelaxation factor [5]. Moreover, the degraded compounds of heme can be expected to facilitate Fenton reactions to induce peroxidation of unsaturated lipids in cell membranes [6]. These potential toxicities imply the physiological importance of cellular membranes of RBCs for encapsulation. They also show a capability of mimicking the cellular structure for producing Hb-based oxygen carriers (HBOCs). Ultrathin membranes of synthetic polymer and cross-linked protein membrane artificial red blood cells containing Hb and enzymes were prepared in 1950s by Thomas Chang of McGill Univ., and by other groups. Studies of encapsulation of functional molecules with phospholipids started after the discovery of liposomes by Bangham in the 1960s. Djordjevici and Miller in 1977 first reported liposome encapsulated Hb (LEH). Many research groups have since used different lipid species and compositions as attempts to encapsulate Hb using liposomes and to improve their encapsulation efficiency, biocompatibility, stability during storage, and oxygen-carrying capacity [7]. Because of the difficulty in resolving the issues presented above, most groups eventually terminated their development. However, we have continued the research and development of hemoglobin vesicles (HbV) and have clarified their safety and efficacy through abundant preclinical studies. After having full consultation with Pharmaceuticals and Medical Devices Agency (PMDA) and achieving GLP preclinical studies, our academic consortium initiated a phase 1 (first-in-human) study of HbV in 2020.

Preparation of Encapsulated Hb Using Liposomes

The research and development of HBOCs in Japan began in the 1980s with the concept of recycling of unused donated blood. Pyridoxalated Hb polyoxyethylene conjugate (PHP) produced by Ajinomoto Co. Inc. and Neo Red Cells (NRC) produced by Terumo Corp. were developed at that time.

H. Sakai (✉) · N. Kobayashi · T. Kure
Department of Chemistry, Nara Medical University, Kashihara, Japan
e-mail: hirosakai@naramed-u.ac.jp

H. Azuma
Department of Pediatrics, Asahikawa Medical University, Asahikawa, Japan

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_22

Because the intraerythrocytic components are not only Hb but also glycolytic enzymes, carbonic anhydrase, metHb reducing enzymes, catalase, superoxide dismutase, etc., some groups tried to preserve such enzymes. However, these enzymes are generally unstable. The enzymatic activities cannot be preserved during an Hb purification process and during a long storage period. Our present concept is to eliminate such unstable enzymes during virus inactivation/removal processes for the utmost safety from infection, even though the donated blood was confirmed as virus-free through specific nucleic acid amplification tests [7]. The processes of Hb purification from outdated donated human blood includes procedures of pasteurization (60 °C, 12 h) and nanofiltration [8], respectively, for virus inactivation and removal. For such purposes, carbonylation of Hb in advance to produce carbonylhemoglobin (HbCO) is effective. HbCO is thermally stable for pasteurization. It can be stored for a long time as a starting material of HBOC production.

We have so many selections of lipids as amphiphiles for Hb encapsulation, such as phospholipids (with various polar head groups, and esterified with various length of saturated or unsaturated fatty acids), cholesterol, and surface modifiers (polymers, charges), as natural or synthetic products, but the lipids should be selected carefully in terms of the encapsulation efficiency, stability during storage and blood circulation, and biocompatibility after intravenous administration. Even though encapsulation can shield the toxicity of molecular Hb, one must be careful about the biocompatibility of the materials for encapsulation. Actually, encapsulated Hb using polymerized phospholipid showed enormous stability during storage, but it did not decompose in reticuloendothelial system (RES) for a long time. Encapsulated Hbs with liposomes containing negatively charged phosphatidyl glycerol or fatty acid induced complement and platelet activation [7]. For Hb encapsulation, we selected a mixture of four lipids: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, 1,5-*O*-dihexadecyl-L-glutamate, and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} [6]. The oxygen affinity (P_{50}) of HbV is adjusted, if necessary, by co-encapsulation of an allosteric effector, pyridoxal 5′-phosphate (PLP), to 9–30 Torr, depending on the usage. Currently, we produce HbV with P_{50} = 15 mmHg by co-encapsulating equimolar PLP to Hb. Therefore, the oxygen dissociation curve is left-shifted in comparison to human RBCs. This formulation is effective for targeted oxygen delivery to tissues where oxygen is needed. The particle size is adjusted to 250–280 nm by extrusion or kneading [9]. The spherical unilamellar structure encapsulating a concentrated Hb solution is confirmed by small-angle X-ray scattering [10]. Actually, HbCO can be converted to HbO_2 by illumination with visible light under an aerobic atmosphere [11]. Finally, the deoxygenated HbV is purged with nitrogen in vials or in plastic bags sealed in aluminum bags for long-term storage. Recent studies have clarified that HbV is useful as a CO carrier for anti-inflammatory and anti-oxidative effects in some pathological conditions. For such uses, carbonyl state CO-HbV without the processes of decarbonylation and deoxygenation is purged with CO gas (Fig. 22.1). A considerable difference exists in colloid osmotic pressure (COP), across the liposomal membrane, between outer saline medium (COP = 0 Torr) and inner concentrated Hb solution (COP = ca. 254 Torr). That pressure difference slightly affects the liposomal membrane fluidity. However, the spherical structure is preserved for a long time without causing hemolysis [12]. The Hb concentration is adjusted to 10 g/dL, which is slightly lower than that of human blood (12–15 g/dL), but much higher than the transfusion threshold, which is known to be 6–7 g/dL in critical patient blood [13]. Usually, HbV is suspended in a physiological saline solution. The outstanding difference of HbV in comparison to chemically modified Hb solutions is the absence of colloid osmotic pressure. These are the same characteristics of RBCs. Some chemically modified Hb solutions exceed the physiological colloid osmotic pressure (20–25 Torr), limiting their Hb concentration. HbV can be suspended in or co-injected with colloidal solutions such as human serum albumin, HES, or modified fluid gelatin solutions to adjust the colloid osmotic pressure [14]. The percentage of the occupied volume of the HbV particles corresponds to about 40–45% (cf., hematocrit of blood is about 40–55%). Therefore, the suspension is a concentrated particle dispersion, similar to RBCs in blood. A certain level of viscosity is expected to be important for inducing shear stress on the vascular wall to facilitate vasorelaxation and blood flow [15].

Potential Clinical Applications of HbV as a Transfusion Alternative

The first distinguished animal experiment of HbV was testing extreme hemodilution up to 90% blood exchange (hematocrit reduction from 50% to 5%) using rats by repeated 1 mL blood withdrawal and 1 mL injection of HbV suspended in 5% albumin, which showed stable hemodynamic and blood gas parameters and tissue oxygenation [16, 17]. These results assured the sufficient oxygen carrying capacity of HbV and became the driving force for HbV R&D.

The ultimate usage of HbV will be to save lives of patients suffering from massive hemorrhage where blood is not available. We conducted various studies of resuscitation from hemorrhagic shock using rodents, rabbits, and beagle dogs by injection of HbV suspended in 5% albumin [3]. The beagle dogs survived for over 1 year and the rats for over 14 days after resuscitation from hemorrhagic shock without major

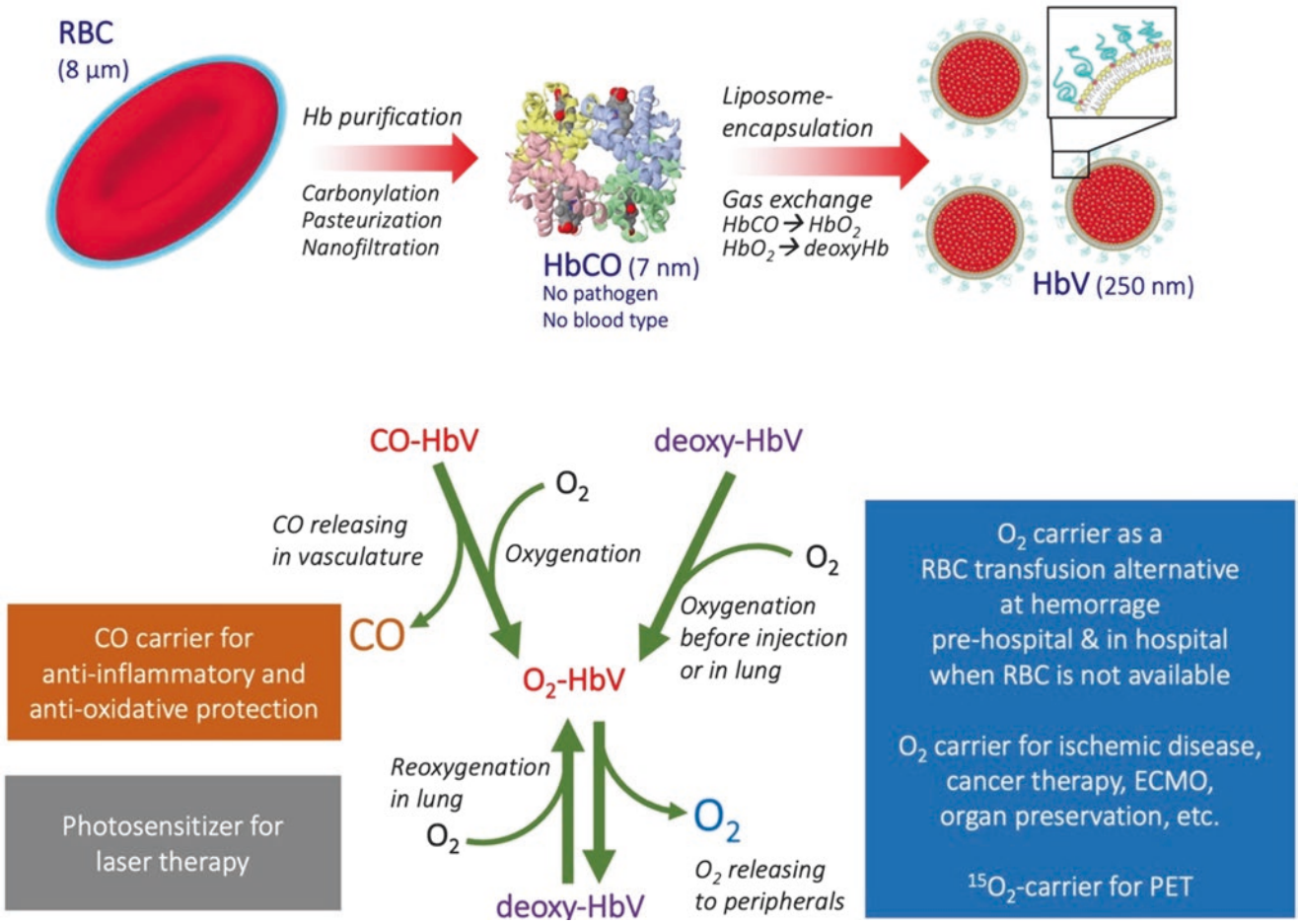


Fig. 22.1 Preparation of hemoglobin vesicles (HbVs) as oxygen and carbon monoxide carriers and a photosensitizer for various clinical applications

side effects except the transient splenomegaly, with an increase in plasma cholesterol levels attributable to the RES trap of HbV and succeeding degradation [18].

Recent studies showed that one-year-stored HbV showed effective resuscitation without showing acute lung injury [19]. Continuous injection of HbV into rats with hemorrhagic shock and uncontrolled hemorrhage, mimicking a condition of prehospital treatment, showed survival even after the hematocrit decreased to less than 2% [20]. Hemorrhagic-shocked rabbits with thrombocytopenic coagulopathy were resuscitated using HbV, with transfusion of platelet rich plasma or artificial platelets. The combination of the oxygen-carrying fluid and hemostatic agent showed effective resuscitative effects without disturbing hemostasis [21, 22]. The small HbV (250 nm) has been proven effective for urgent resuscitation through intraosseous injection when peripheral vessels are collapsed and inaccessible [23]. In addition, HbV infusion has been confirmed as effective for the initial management of massive obstetric hemorrhage in a pregnant rabbit model [24].

Assuming a peri-operational usage of HbV, a pneumonectomy model with hemorrhage of about 30–40% blood volume was prepared using mice and rats; HbV with 5% albumin was injected intravenously [25, 26]. The oxygen-carrying capability of HbV was comparable to that of rodent RBCs, even under impaired lung function after pneumonectomy. In fact, HbV with a high oxygen affinity might have more beneficial effects on oxygenation. Results show that HbV infusion did not interfere with the recovery from surgical injury. Following these promising results, a similar study of pneumonectomy model using beagle dogs is underway by Kohno et al. of Tokai University.

Prolonged hypotension after hemorrhagic shock causes irreversible heart dysfunction called "shock heart syndrome," which is associated with lethal arrhythmias. The standard protocol of hemorrhagic shock resuscitation is to inject crystalloid first, followed by colloid injection, such as HES, gelatin, and dextran, and finally by packed RBC transfusion. This protocol follows a strategy to avoid allogeneic transfusion. However, Takase et al. clarified that the initial resuscitation

with simply crystalloids or colloids, without O_2 carrying capacity, shows higher incidence of lethal arrhythmias than either RBC transfusion or HbV injection. The results indicate that resuscitation with O_2-carrying HbV injection initially should be of primary importance for avoiding shock heart syndrome and for improving the survival rate by preventing electrical remodeling while preserving myocardial structures [27].

Potential Clinical Applications of HbV for Oxygen and Carbon Monoxide Therapeutics

The COVID-19 epidemic has caused grievous damage to pulmonary alveolar structure and subsequent respiratory failure in many patients. Extracorporeal membrane oxygenation (ECMO) has been proven to be an effective therapy for such condition [28]. ECMO requires a fluid to prime the circuit, resulting in dilution of blood. The hemodilution effect increases with smaller body weight. In the case of cardiac surgery using ECMO, the effect on the neurological function of newborn patients is considerable because the decreased oxygen supply during surgery affects brain function. Damage appears after the newborns are grown up. However, observation of a rat model confirmed that ECMO primed with HbV suspended in 5% HSA showed sustained oxygenation and prevented neurocognitive decline, as confirmed with water-maze testing [29]. Moreover, HbV is proven to be effective as an extracorporeal perfusion fluid to carry oxygen for organs for *ex vivo* experimental purpose to observe intestinal peristaltic motion [30]. It is effective for preservation of an amputated rat leg by perfusion with HbV for several hours and then re-implantation to the rat [31]. It is also effective for subnormothermic machine perfusion of pig livers for preservation [32]. HbV is useful as a carrier of $^{15}O_2$ for positron emission tomography (PET). Actually, injection of $^{15}O_2$-HbV into a stroke rat model visualized the lowered oxygen metabolism in the infarcted area in PET images [33].

Because of the smaller size of HbV than of RBCs, the injected HbV particles are distributed homogeneously in the plasma phase. In the microscopic view of microcirculation, plasma skimming is readily observed at a branch of a small artery or arterioles where the hematocrit values of daughter branches differ because of the different blood flow rate in each branch. This condition induces plasma skimming. The branch of a slower blood flow rate shows a lower hematocrit. In such a condition, HbV distributes homogeneously in the plasma phase and distributes more in the branch of a slower blood flow rate. HbV can carry oxygen where RBC flow is limited in the ischemic tissues. It has been demonstrated that intravenous administration of HbV after occlusion of the middle cerebral artery can attenuate the infarction volume [34]. Moreover, HbV can rescue placental hypoxia in a rat pre-eclampsia model [35]. Because of the same mechanism HbV increases the oxygen tension of tumor tissue where the capillary structure is abnormal and RBC flow is considerably limited [36].

HbV is useful not only as an oxygen carrier, but also as a photosensitizer for laser irradiation therapy. Rikihisa et al. reported the utilization of HbV as a photosensitizer, a target of laser treatment of port-wine stain (capillary malformation) [37, 38] because injection of small HbV distributes between the RBCs in capillaries and increases capillary Hb levels effectively, thereby producing more heat and photocoagulation.

Various kinds of CO-releasing molecules (CORM) have been reported: not only metal complexes but also organic compounds [39–41]. They show beneficial cytoprotective effects in animal models of septic shock and ischemia reperfusion injury, and inflammatory diseases. These results were the driving force to test the CO-bound HbV (CO-HbV) as another type of CORM. In fact, CO-HbV was first tested for resuscitation from hemorrhagic shock in a rat model [42]. The blood HbCO increased to about 30% immediately after injection, but it decreased in 3 h. Also, the dissociated CO appeared in the exhaled air simultaneously. Plasma enzyme levels, AST and ALT, were lower than those associated with O_2-HbV injection, indicating that CO ameliorated reperfusion injury. Taguchi et al. applied CO-HbV for models of bleomycin-induced pulmonary fibrosis [43], dextran sulfate sodium-induced colitis [44], and acute pancreatitis [45]. Liberated CO showed marked anti-inflammatory and anti-oxidative properties, probably because of the interaction of CO with hemeproteins related to the production of reactive oxygen and nitrogen species in the body under pathological conditions.

From the viewpoint of production, CO-bound HbV can be produced more easily than deoxygenated HbV because the processes of decarbonylation and deoxygenation are not necessary. After releasing CO in blood circulation, HbV reversibly binds O_2. It thereby becomes an oxygen carrier. CO-HbV is expected to provide unique opportunities for the clinical treatment of various pathological conditions. However, additional studies must be conducted to confirm the absence of the chronic neurological toxicity of CO because CO is known fundamentally as a toxic gas. Its neurological effects are known to manifest later.

Safety Evaluations of HbV

Encapsulation of Hb can shield the toxic effects of Hb. Nevertheless, one must be careful about the toxicity of the capsules and of the liposomal lipid composition. One outstanding advantage of HbV in comparison to conventional

liposome-encapsulated Hb is the absence of complement activation and anaphylactoid reaction, as confirmed through a pig study [46]. Even though the conventional liposomes showed a considerable increase in the pulmonary vascular resistance at repeated injections, HbV with optimized lipid composition showed no such reaction. Preclinical studies of HbV show no significant effect on the complement system, immunological response, blood coagulation, platelet function, kallikrein–kinin, hematopoiesis, etc. [47]. The HbV particles are finally captured by the RES or mononuclear phagocytic system (MPS). Therefore, transient hepatosplenomegaly is observed depending on the HbV dosage [18]. However, the HbV components are degraded completely in RES, and are eliminated and excreted through urine and feces [48]. Our studies using rodent models showed that the macrophages capturing the liposomes become similar to myeloid-derived suppressor cells (MDSCs) which cause suppression of splenic T cell proliferation [49, 50], though the response is transiently observed and its effect is minimal. Reportedly recent studies of PEGylated liposomes for cancer therapy and other PEGylated materials clarified that the presence of anti-PEG antibody is frequently observed in humans. It might change the pharmacokinetics of the agents (ABC phenomenon) [48] or induce immunological reactions [51]. This point is expected to demand some attention in the ongoing clinical research and development of HbV.

Summary

Including liposome-encapsulated Hb, recent trends in the development of HBOCs are to make them larger [52–60] to prevent extravasation, to retard reactions with NO [61], and to lower the colloid osmotic pressure [62]. However, not only the particle design, but also the design of its suspension in terms of physicochemical properties such as Hb concentration, colloid osmotic pressure, and viscosity are important factors that must be examined to make the fluid function as a blood substitute. As described in this chapter, we summarized potential clinical applications of HbV evidenced using various animal experimental models. Of course, other HBOCs have some potential to be used in the same manner.

An ultimate and optimal usage of HBOCs, taking the advantages of their characteristics superior to packed RBCs, should be for saving lives of lethal patients of massive bleeding and trauma when blood for transfusion is not available. However, it is difficult to obtain an informed consent from a lethal patient. Accordingly, clinical studies in the next stage would be limited to injections to patients with perioperational bleeding at elective surgeries or patients with post-operational anemia. A success of such study will eventually enable various usage of HBOCs for any purposes.

Key Points

- Encapsulated Hbs, including HbV, have been designed to mimic the cellular structure of RBCs for eliminating the toxic effect of molecular Hbs.
- However, it is important to consider the capsule material safety as well, especially on the absence of complement activation, dispersibility in blood, and degradation in RES.
- Safety and efficacy of HbV as a transfusion alternative have been evaluated in hemorrhagic shock-resuscitation preclinical studies.
- HbV has been tested not only as a transfusion alternative, but also as a new agent for oxygen therapeutics, a perfusate for organ preservation, and a photosensitizer for laser therapy.
- CO-HbV liberates CO in blood circulation and shows anti-oxidative and anti-inflammatory effects. CO-HbV will be a new promising agent though its neurological effect has to be examined carefully.

Acknowledgments The authors extend deep appreciation to supervisors and all collaborators in the academic research consortium of HbV who contributed to the production and the safety and efficacy evaluations of HbV. This research has been supported partly by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Sciences, a Health, Labour and Sciences Research Grant from the Ministry and Health, Labour and Welfare, Japan, and the Project Promoting Clinical Trials for Development of New Drugs and Medical Devices from the Japan Agency for Medical Research and Development, AMED.

Conflict of Interest Of the authors, H.S. is a holder of the patents related to the production and utilization of HbV.

References

1. Ngo A, Masel D, Cahill C, Blumberg N, Refaai MA. Blood banking and transfusion medicine challenges during the COVID-19 pandemic. Clin Lab Med. 2020;40(4):587–601. https://doi.org/10.1016/j.cll.2020.08.013.
2. Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E. Haemoglobin-vesicles as artificial oxygen carriers: present situation and future visions. J Intern Med. 2008;263(1):4–15. https://doi.org/10.1111/j.1365-2796.2007.01893.x.
3. Sakai H. Overview of potential clinical applications of hemoglobin vesicles (HbV) as artificial red cells, evidenced by preclinical studies of the academic research consortium. J Funct Biomater. 2017;8(1):10. https://doi.org/10.3390/jfb8010010.
4. Matsuhira T, Kure T, Yamamoto K, Sakai H. Analysis of dimeric αβ subunit exchange between PEGylated and native hemoglobins ($\alpha_2\beta_2$ tetramer) in an equilibrated state by intramolecular ββ-cross-linking. Biomacromolecules. 2018;19(8):3412–20. https://doi.org/10.1021/acs.biomac.8b00728.
5. Winslow R. Hemoglobin-based red cell substitute. Baltimore: The John Hopkins University Press; 1992.
6. Takeoka S, Teramura Y, Atoji T, Tsuchida E. Effect of Hb-encapsulation with vesicles on H_2O_2 reaction and lipid peroxidation. Bioconjug Chem. 2002;13(6):1302–8. https://doi.org/10.1021/bc025546k.

7. Sakai H, Sou K, Tsuchida E. Hemoglobin-vesicles as an artificial oxygen carrier. Methods Enzymol. 2009;465:363–84. https://doi.org/10.1016/S0076-6879(09)65019-9.
8. Naito Y, Fukutomi I, Masada Y, Sakai H, Takeoka S, Tsuchida E, Abe H, Hirayama J, Ikebuchi K, Ikeda H. Virus removal from hemoglobin solution using Planova membrane. J Artif Organs. 2002;5:141–5. https://doi.org/10.1007/s100470200025.
9. Kure T, Sakai H. Preparation of artificial red blood cells (hemoglobin vesicles) using rotation-revolution mixer for high encapsulation efficiency. ACS Biomater Sci Eng. 2021;7(6):2835–44. https://doi.org/10.1021/acsbiomaterials.1c00424.
10. Sato T, Sakai H, Sou K, Medebach M, Glatter O, Tsuchida E. Static structures and dynamics of hemoglobin vesicle (HBV) developed as a transfusion alternative. J Phys Chem B. 2009;113(24):8418–28. https://doi.org/10.1021/jp9002142.
11. Chung JE, Hamada K, Sakai H, Takeoka S, Nishide H, Tsuchida E. Ligand exchange reaction of carbonylhemoglobin to oxyhemoglobin in a hemoglobin liquid membrane. Nihonkagakukaishi. 1995:123–7. (in Japanese). https://doi.org/10.1246/nikkashi.1995.123.
12. Kure T, Sakai H. Transmembrane difference in colloid osmotic pressure affects the lipid membrane fluidity of liposomes encapsulating a concentrated protein solution. Langmuir. 2017;33(6):1533–40. https://doi.org/10.1021/acs.langmuir.6b04643.
13. Carson JL, Carless PA, Hébert PC. Outcomes using lower vs higher hemoglobin thresholds for red blood cell transfusion. JAMA. 2013;309(1):83–4. https://doi.org/10.1001/jama.2012.50429.
14. Sakai H, Miyagawa N, Horinouchi H, Takeoka S, Takaori M, Tsuchida E, Kobayashi K. Intravenous infusion of Hb-vesicles (artificial oxygen carriers) after repetitive blood exchange with a series of plasma expanders (water-soluble biopolymers) in a rat model. Polymer Adv Technol. 2011;22(8):1216–22. https://doi.org/10.1002/pat.1964.
15. Tsai AG, Friesenecker B, McCarthy M, Sakai H, Intaglietta M. Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skinfold model. Am J Phys. 1998;275(6):H2170–80. https://doi.org/10.1152/ajpheart.1998.275.6.H2170.
16. Izumi Y, Sakai H, Hamada K, Takeoka S, Yamahata T, Kato R, Nishide H, Tsuchida E, Kobayashi K. Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical tissue oxygen tension. Crit Care Med. 1996;24(11):1869–73. https://doi.org/10.1097/00003246-199611000-00017.
17. Sakai H, Takeoka S, Park S, Kose T, Nishide H, Izumi Y, Yoshizu A, Kobayashi K, Tsuchida E. Surface modification of hemoglobin vesicles with poly(ethylene glycol) and affects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. Bioconjug Chem. 1997;8:23–30. https://doi.org/10.1021/bc960069p.
18. Ikeda T, Horinouchi H, Izumi Y, Sakai H, Kobayashi K. Chapter 28: Cellular-type hemoglobin-based oxygen carrier as a resuscitative fluid for hemorrhagic shock: acute and long-term safety evaluation using beagle dogs. In: Kim HW, Greenburg AG, editors. Hemoglobin-based oxygen carriers as red cell substitutes and oxygen therapeutics. Berlin: Springer-Verlag Berlin Heidelberg; 2013. p. 501–24. https://doi.org/10.1007/978-3-642-40717-8_28.
19. Tokuno M, Taguchi K, Yamasaki K, Sakai H, Otagiri M. Long-term stored hemoglobin-vesicles, a cellular type of hemoglobin-based oxygen carrier, has resuscitative effects comparable to that for fresh red blood cells in a rat model with massive hemorrhage without post-transfusion lung injury. PLoS One. 2016;11:e0165557. https://doi.org/10.1371/journal.pone.0165557.
20. Seishi Y, Horinouchi H, Sakai H, Kobayashi K. Effect of the cellular-type artificial oxygen carrier hemoglobin vesicle as a resuscitative fluid for prehospital treatment: experiments in a rat uncontrolled hemorrhagic shock model. Shock. 2012;38:153–8. https://doi.org/10.1097/SHK.0b013e31825ad7cf.
21. Hagisawa K, Kinoshita M, Takase B, Hashimoto K, Saitoh D, Seki S, Nishida Y, Sakai H. Efficacy of resuscitative transfusion with hemoglobin vesicles in the treatment of massive hemorrhage in rabbits with thrombocytopenic coagulopathy and its effect on hemostasis by platelet transfusion. Shock. 2018;50(3):324–30. https://doi.org/10.1097/SHK.0000000000001042.
22. Hagisawa K, Kinoshita M, Takikawa M, Takeoka S, Saitoh D, Seki S, Sakai H. Combination therapy using fibrinogen γ-chain peptide-coated, ADP-encapsulated liposomes and hemoglobin vesicles for trauma-induced massive hemorrhage in thrombocytopenic rabbits. Transfusion. 2019;59(10):3186–96. https://doi.org/10.1111/trf.15427.
23. Hagisawa K, Kinoshita M, Saitoh D, Morimoto Y, Sakai H. Intraosseous transfusion of hemoglobin vesicles in the treatment of hemorrhagic shock with collapsed vessels in a rabbit model. Transfusion. 2020;60(7):1400–9. https://doi.org/10.1111/trf.15915.
24. Yuki Y, Hagisawa K, Kinoshita M, Ishibashi H, Kaneko K, Ishida O, Saitoh D, Sakai H, Terui K. Efficacy of resuscitative infusion with hemoglobin vesicles in rabbits with massive obstetric hemorrhage. Am J Obstet Gynecol. 2020:S0002-9378(20)31067-X. https://doi.org/10.1016/j.ajog.2020.09.010.
25. Takase B, Higashimura Y, Hashimoto K, Asahina H, Ishihara M, Sakai H. Myocardial electrical remodeling and the arrhythmogenic substrate in hemorrhagic shock-induced heart: anti-arrhythmogenic effect of liposome-encapsulated hemoglobin (HbV) on the myocardium. Shock. 2019;52(3):378–86. https://doi.org/10.1097/SHK.0000000000001262.
26. Kohno M, Ikeda T, Hashimoto R, Izumi Y, Watanabe M, Horinouchi H, Sakai H, Kobayashi K, Iwazaki M. Acute 40% exchange-transfusion with hemoglobin-vesicles in a mouse pneumonectomy model. PLoS One. 2017;12(6):e0178724. https://doi.org/10.1371/journal.pone.0178724.
27. Hashimoto R, Kohno M, Oiwa K, Onozawa H, Watanabe M, Horinouchi H, Sakai H, Kobayashi K, Iwazaki M. Immediate effects of systemic administration of normal and high O_2-affinity haemoglobin vesicles as a transfusion alternative in a rat pneumonectomy model. BMJ Open Respir Res. 2020;7(1):e000476. https://doi.org/10.1136/bmjresp-2019-000476.
28. Salas de Armas IA, Akkanti BH, Janowiak L, Banjac I, Dinh K, Hussain R, Cabrera R, Herrera T, Sanger D, Akay MH, Patel J, Patel MK, Kumar S, Jumean M, Kar B, Gregoric ID. Inter-hospital COVID ECMO air transportation. Perfusion. 2020;267659120973843 https://doi.org/10.1177/0267659120973843.
29. Yamazaki M, Aeba R, Yozu R, Kobayashi K. Use of hemoglobin vesicles during cardiopulmonary bypass priming prevents neurocognitive decline in rats. Circulation. 2006;114(1 Suppl):I220–5. https://doi.org/10.1161/CIRCULATIONAHA.105.000562.
30. Verdu EF, Bercik P, Huang XX, Lu J, Al-Mutawaly N, Sakai H, Tompkins TA, Croitoru K, Tsuchida E, Perdue M, Collins SM. The role of luminal factors in the recovery of gastric function and behavioral changes after chronic *Helicobacter pylori* infection. Am J Physiol Gastrointest Liver Physiol. 2008;295(4):G664–70. https://doi.org/10.1152/ajpgi.90316.2008.
31. Araki J, Sakai H, Takeuchi D, Kagaya Y, Tashiro K, Naito M, Mihara M, Narushima M, Iida T, Koshima I. Normothermic preservation of the rat hind limb with artificial oxygen-carrying hemoglobin vesicles. Transplantation. 2015;99(4):687–92. https://doi.org/10.1097/TP.0000000000000528.
32. Shonaka T, Matsuno N, Obara H, Yoshikawa R, Nishikawa Y, Ishihara Y, Bochimoto H, Gochi M, Otani M, Kanazawa H, Azuma H, Sakai H, Furukawa H. Impact of human-derived

hemoglobin-based oxygen vesicles as a machine perfusion solution for liver donation after cardiac death in a pig model. PLoS One. 2019;14(12):e0226183. https://doi.org/10.1371/journal.pone.0226183.
33. Tiwari VN, Kiyono Y, Kobayashi M, Mori T, Kudo T, Okazawa H, Fujibayashi Y. Automatic labeling method for injectable ^{15}O-oxygen using hemoglobin-containing liposome vesicles and its application for measurement of brain oxygen consumption by PET. Nucl Med Biol. 2010;37(1):77–83. https://doi.org/10.1016/j.nucmedbio.2009.08.004.
34. Komatsu H, Furuya T, Sato N, Ohta K, Matsuura A, Ohmura T, Takagi S, Matsuura M, Yamashita M, Itoda M, Itoh J, Horinouchi H, Kobayashi K. Effect of hemoglobin vesicle, a cellular-type artificial oxygen carrier, on middle cerebral artery occlusion- and arachidonic acid-induced stroke models in rats. Neurosci Lett. 2007;421(2):121–5. https://doi.org/10.1016/j.neulet.2007.04.080.
35. Li H, Ohta H, Tahara Y, Nakamura S, Taguchi K, Nakagawa M, Oishi Y, Goto Y, Wada K, Kaga M, Inagaki M, Otagiri M, Yokota H, Shibata S, Sakai H, Okamura K, Yaegashi N. Artificial oxygen carriers rescue placental hypoxia and improve fetal development in the rat pre-eclampsia model. Sci Rep. 2015;5:15271. https://doi.org/10.1038/srep15271.
36. Yamamoto M, Izumi Y, Horinouchi H, Teramura Y, Sakai H, Kohno M, Watanabe M, Kawamura M, Adachi T, Ikeda E, Takeoka S, Tsuchida E, Kobayashi K. Systemic administration of hemoglobin vesicle elevates tumor tissue oxygen tension and modifies tumor response to irradiation. J Surg Res. 2009;151(1):48–54. https://doi.org/10.1016/j.jss.2007.12.770.
37. Rikihisa N, Watanabe S, Satoh K, Saito Y, Sakai H. Photosensitizer effects of artificial red cells on dye laser irradiation in an animal model assuming port-wine stain treatment. Plast Reconstr Surg. 2017;139(3):707e–16e. https://doi.org/10.1097/PRS.0000000000003082.
38. Rikihisa N, Tominaga M, Watanabe S, Mitsukawa N, Saito Y, Sakai H. Intravenous injection of artificial red cells and subsequent dye laser irradiation causes deep vessel impairment in an animal model of port-wine stain. Lasers Med Sci. 2018;33(6):1287–93. https://doi.org/10.1007/s10103-018-2480-2.
39. Vummaleti SV, Branduardi D, Masetti M, De Vivo M, Motterlini R, Cavalli A. Theoretical insights into the mechanism of carbon monoxide (CO) release from CO-releasing molecules. Chemistry. 2012;18(30):9267–75. https://doi.org/10.1002/chem.201103617.
40. Nguyen D, Boyer C. Macromolecular and inorganic nanomaterials scaffolds for carbon monoxide delivery: recent developments and future trends. ACS Biomater Sci Eng. 2015;1(10):895–913. https://doi.org/10.1021/acsbiomaterials.5b00230.
41. Lazarus LS, Benninghoff AD, Berreau LM. Development of triggerable, trackable, and targetable carbon monoxide releasing molecules. Acc Chem Res. 2020;53(10):2273–85. https://doi.org/10.1021/acs.accounts.0c00402.
42. Sakai H, Horinouchi H, Tsuchida E, Kobayashi K. Hemoglobin vesicles and red blood cells as carriers of carbon monoxide prior to oxygen for resuscitation after hemorrhagic shock in a rat model. Shock. 2009;31(5):507–14. https://doi.org/10.1097/SHK.0b013e318188f83d.
43. Nagao S, Taguchi K, Sakai H, Tanaka R, Horinouchi H, Watanabe H, Kobayashi K, Otagiri M, Maruyama T. Carbon monoxide-bound hemoglobin-vesicles for the treatment of bleomycin-induced pulmonary fibrosis. Biomaterials. 2014;35(24):6553–62. https://doi.org/10.1016/j.biomaterials.2014.04.049.
44. Nagao S, Taguchi K, Miyazaki Y, Wakayama T, Chuang VT, Yamasaki K, Watanabe H, Sakai H, Otagiri M, Maruyama T. Evaluation of a new type of nano-sized carbon monoxide donor on treating mice with experimentally induced colitis. J Control Release. 2016;234:49–58. https://doi.org/10.1016/j.jconrel.2016.05.016.
45. Taguchi K, Nagao S, Maeda H, Yanagisawa H, Sakai H, Yamasaki K, Wakayama T, Watanabe H, Otagiri M, Maruyama T. Biomimetic carbon monoxide delivery based on hemoglobin vesicles ameliorates acute pancreatitis in mice via the regulation of macrophage and neutrophil activity. Drug Deliv. 2018;25(1):1266–74. https://doi.org/10.1080/10717544.2018.1477860.
46. Sakai H, Suzuki Y, Sou K, Kano M. Cardiopulmonary hemodynamic responses to the small injection of hemoglobin vesicles (artificial oxygen carriers) in miniature pigs. J Biomed Mater Res A. 2012;100(10):2668–77. https://doi.org/10.1002/jbm.a.34208.
47. Azuma H, Fujihara M, Sakai H. Chapter 22: Biocompatibility of hemoglobin vesicles, a cellular-type artificial oxygen carrier, on blood cells and plasma proteins in vitro and in vivo. In: Kim HW, Greenburg AD, editors. Hemoglobin-based oxygen carriers as red cell substitutes and oxygen therapeutics. Berlin Heidelberg: Springer-Verlag; 2013. p. 385–97. https://doi.org/10.1007/978-3-642-40717-8_22.
48. Taguchi K, Urata Y, Anraku M, Maruyama T, Watanabe H, Sakai H, Horinouchi H, Kobayashi K, Tsuchida E, Kai T, Otagiri M. Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. Drug Metab Dispos. 2009;37(7):1456–63. https://doi.org/10.1124/dmd.109.027094.
49. Azuma H, Fujihara M, Sakai H. Biocompatibility of HbV: liposome-encapsulated hemoglobin molecules-liposome effects on immune function. J Funct Biomater. 2017;8(3):24. https://doi.org/10.3390/jfb8030024.
50. Yoshida Y, Nagamori T, Ishibazawa E, Kobayashi H, Kure T, Sakai H, Takahashi D, Fujihara M, Azuma H. Contribution of long-chain fatty acid to induction of myeloid-derived suppressor cell (MDSC)-like cells - induction of MDSC by lipid vesicles (liposome). Immunopharmacol Immunotoxicol. 2020;42(6):614–24. https://doi.org/10.1080/08923973.2020.1837866.
51. d'Avanzo N, Celia C, Barone A, Carafa M, Di Marzio L. Immunogenicity of polyethylene glycol based nanomedicines: mechanisms, clinical implications and systematic approach. Adv Therap. 2020. https://doi.org/10.1002/ADTP.201900170.
52. Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. Am J Physiol Heart Circ Physiol. 2000;279(3):H908–15. https://doi.org/10.1152/ajpheart.2000.279.3.H908.
53. Sen Gupta A, Doctor A. Chapter 11: Oxygen carriers. In: Spinella PC, editor. Damage control resuscitation. Cham: Springer Nature Switzerland G; 2020. p. 197–222.
54. Yadav VR, Rao G, Houson H, Hedrick A, Awasthi S, Roberts PR, Awasthi V. Nanovesicular liposome-encapsulated hemoglobin (LEH) prevents multi-organ injuries in a rat model of hemorrhagic shock. Eur J Pharm Sci. 2016;93:97–106. https://doi.org/10.1016/j.ejps.2016.08.010.
55. Jansman MMT, Liu X, Kempen P, Clergeaud G, Andresen TL, Thulstrup PW, Hosta-Rigau L. Hemoglobin-based oxygen carriers incorporating nanozymes for the depletion of reactive oxygen species. ACS Appl Mater Interfaces. 2020;12(45):50275–86. https://doi.org/10.1021/acsami.0c14822.
56. Guo J, Agola JO, Serda R, Franco S, Lei Q, Wang L, Minster J, Croissant JG, Butler KS, Zhu W, Brinker CJ. Biomimetic rebuilding of multifunctional red blood cells: modular design using functional components. ACS Nano. 2020;14(7):7847–59. https://doi.org/10.1021/acsnano.9b08714.
57. Matsuhira T, Yamamoto K, Sakai H. Ring-opening polymerization of hemoglobin. Biomacromolecules. 2019;20(4):1592–602. https://doi.org/10.1021/acs.biomac.8b01789.
58. Hickey R, Palmer AF. Synthesis of hemoglobin-based oxygen carrier nanoparticles by desolvation precipitation.

Langmuir. 2020;36(47):14166–72. https://doi.org/10.1021/acs.langmuir.0c01698.
59. Kao I, Xiong Y, Steffen A, Smuda K, Zhao L, Georgieva R, Pruss A, Bäumler H. Preclinical in vitro safety investigations of submicron sized hemoglobin based oxygen carrier HbMP-700. Artif Organs. 2018;42(5):549–59. https://doi.org/10.1111/aor.13071.
60. Wang Y, Yan L, He S, Zhou D, Cheng Y, Chen X, Jing X, Huang Y. A versatile method to prepare protein nanoclusters for drug delivery. Macromol Biosci. 2018;18(2). https://doi.org/10.1002/mabi.201700282.
61. Cardenas ASB, Samuel PP, Olson JS. Current challenges in the development of acellular hemoglobin oxygen carriers by protein engineering. Shock. 2019;52(1S Suppl 1):28–40. https://doi.org/10.1097/SHK.0000000000001053.
62. Sakai H, Yuasa M, Onuma H, Takeoka S, Tsuchida E. Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. Bioconjug Chem. 2000;11(1):56–64. https://doi.org/10.1021/bc9900789.

23

Potential Value of Polynitroxylated PEGylated Hemoglobin (SanFlow) in Pre-Hospital Medicine in Austere Environments including Military Deployments, Disasters and Remote Emergencies

William G. Day, Bohdan J. Soltys, Jacob Cole, Jan Simoni, Carleton J. C. Hsia, and Andrew H. Lin

Introduction

Noncompressible hemorrhage continues to be the leading cause of preventable death for warfighters. During the U.S. conflicts between Afghanistan and Iraq from 2001 to 2011, 90 percent of combat casualties died before reaching appropriate surgical care, and 90 percent of those deaths that were survivable were due to uncontrolled hemorrhage [1]. In addition, a significant number of deaths due to hemorrhagic shock occur within the first hour of injury [2]. Recognizing that early intervention is critical to saving lives, the Joint Trauma System and Tactical Combat Casualty Care have focused on point-of-injury hemostasis, early medical evacuation, and damage control resuscitation (DCR) to stabilize casualties prior to definitive surgical care [3].

Advancements in the resuscitation of traumatic hemorrhagic shock have coincided with times of increased armed conflict: the first blood banks were established during WWI, the first use of lyophilized plasma occurred during WWII, the lethal triad of trauma began to take root as a cornerstone of resuscitation during the Vietnam War, and, finally, DCR became the gold standard of early hemorrhage management following the conflicts in Afghanistan and Iraq [4]. As the management of traumatic hemorrhage has evolved over the decades, the paradigm of resuscitation has also shifted from simple volume replacement to a more targeted correction of the physiologic derangements associated with shock.

Running in parallel with advancements in the paradigm of trauma resuscitation are the changes in specific fluid resuscitative strategy, particularly with regards to the type, quantity, and timing of fluid therapy. Traditional resuscitation fluids include the broad categories of crystalloids and colloids. These were the mainstay of traumatic resuscitation for many years in large part due to low cost of production, long-shelf life, lack of need for refrigeration, and generally safe profile. Despite these advantages, complications involving extravascular migration of the fluids into interstitial tissues and dilutional coagulopathy, in concert with the advent of whole blood transfusion, has led to transfusion of whole blood or blood components as the gold standard in DCR [5]. Whole blood transfusion not only provides repletion of intravascular circulating volume, but also provides vital oxygen-carrying capability, clotting factors, and platelets that are crucial for the maintenance of end organ perfusion during life-threatening traumatic hemorrhage.

However, blood transfusion is not without significant risks that must be considered. Transfusion of allogenic blood products can cause immune dysregulation, and issues with immunologic incompatibility can cause a myriad of potentially life-threatening transfusion reactions. Additionally, the logistical and practical challenges of storage, transport, and

Bohdan J. Soltys and Andrew H. Lin contributed equally with all other contributors.

W. G. Day
Department of Internal Medicine, Naval Medical Center Portsmouth, Portsmouth, VA, USA

B. J. Soltys (✉) · C. J. C. Hsia
AntiRadical Therapeutics, LLC, Sioux Falls, SD, USA

AntiRadical Therapeutics Canada Inc., Rosseau, ON, Canada
e-mail: bohdan.soltys@antiradicaltherapeutics.com; chsia@antiradicaltherapeutics.com

J. Cole
Department of Anesthesiology, Naval Medical Center Portsmouth, Portsmouth, VA, USA

J. Simoni
AntiRadical Therapeutics, LLC, Sioux Falls, SD, USA
e-mail: jan.Simoni@ttuhsc.edu

A. H. Lin (✉)
Department of Cardiology, Naval Medical Center Portsmouth, Portsmouth, VA, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_23

supply of blood products limit the ability of forward-deployed combat medics to administer blood products in austere environments [6].

Given these limitations, there has been recent interest in the development of small-volume, artificial oxygen carriers (AOC) that have the ability to adequately resuscitate warfighters in addition to oxygenating tissues. The ideal AOC would approximate colloidal resuscitative fluids of the past such as 5% albumin, and would be able to provide small-volume resuscitation with minimal concern for extravasation from the intravascular space. The wide variety of currently accepted methods for fluid resuscitation and the management of hemorrhagic shock indicates that no single resuscitation strategy is ideal, and there is space in this field for optimization of currently existing resuscitative technologies. Among various AOC molecules developed for use in trauma resuscitation, Polynitroxylated PEGylated Hemoglobin (aka PNPH or SanFlow) is a novel AOC with promising preclinical data suggesting it is an optimal resuscitation fluid. This review aims to provide a critical review of currently developed AOCs with particular emphasis on PNPH including its mechanism of action and the research supporting its potential value for the forward deployed medic responsible for remote DCR.

The Development of Artificial Oxygen Carriers and Hemoglobin Based Oxygen Carriers

Artificial Oxygen Carriers

For more than a century, investigators have sought to produce the ideal resuscitative fluid: a blood substitute that retains all the properties of whole blood without all the inherent dangers and immunologic side effects. Although there has not been a product that has been able to emulate all properties of whole blood, there are compounds that show promising results in two major functional realms: oxygenating tissues and augmenting blood volume. These artificial oxygen carriers (AOC) have been created to reduce whole blood side effects and augment oxygenation of tissues while reducing the volume of fluid required for appropriate resuscitation. To date, there are three main categories of AOC to include Perfluorocarbon Oxygen Carriers (PFOC), stem cells, and Hemoglobin-Based Oxygen Carriers (HBOC), all with varying degrees of *in vitro* and biochemical research [7].

The first potential AOC, PFOC, are fully halogenated molecules that exhibit twice the rate of oxygen unloading as erythrocytes, three times the oxygen extraction rate, and release greater than 90% of the loaded oxygen into tissues. Described side effects of this therapy include lung toxicity, thrombocytopenia, and hypotension. Given these side effects, research has been limited to small biochemical and *in vitro* studies with mixed results [8, 9].

Induced pluripotent stem cells (iPSC), discovered by Shinya Yamanaka in 2006, opened the door for many potential applications for medicine including differentiation into blood components to be stored and used for resuscitation. Two major pathways of differentiation have been considered: differentiation of iPSC to red blood cells and differentiation of iPSC to various target cells in an oxygenated environment. Both avenues have the capability to theoretically reduce the risk of allogenic immunoreactions due to patient-specific production of progenitor cells. However, to date *in vitro* studies have shown issues with retention of erythrocytes, cell deformability, and immunoreactions in allogenic iPSC transfusions [10, 11].

Lastly, Hemoglobin Based Oxygen Carriers (HBOC) are the AOC that have been studied the most extensively. In 1933, Amberson et al. performed studies in cats in which they completely exchanged their blood with cell-free hemoglobin in Lactated Ringer's solution and showed the solution could sustain life. However, the benefit was short lived and the animals experienced significant renal damage [12]. Despite this, human trials commenced which resulted in significant renal toxicity and vascular hypertension in 5 of 14 patients. In the 1950s, the U.S. Navy attempted to treat 47 anemic and febrile patients with cell-free hemoglobin solutions, which again resulted in significant renal toxicity. Renal toxicity during these studies was likely due to a combination of renal tubular obstruction by hemoglobin, renal cell dysfunction due to heme pigment deposition, and hemoglobin-induced vasoconstriction [13].

These pitfalls were eventually resolved through ultra-purification techniques that allowed for removal of cellular debris and stroma. However, a new problem emerged when native tetrameric hemoglobin would breakdown into dimers and be renally extracted, resulting in a vastly reduced half-life of the HBOC. Investigators have since attempted to solve this problem via attaching stabilizing molecules including bis (N-maleimidomethyl) ether (BME), and glutaraldehyde [14].

Limitations of Cell-Free Hemoglobin

Hemoglobin-based oxygen carriers (HBOCs) developed during the past half century and tested in humans have done more harm than good by increasing risk of death and myocardial infarction. This abysmal history, and the resulting negative views of HBOCs held by the medical, regulatory, and investment communities, means that a paradigm shift is

required. The 2008 FDA/NIH co-sponsored HBOC workshop set the view that in order to be successful, an HBOC would have to comply with safety standards in addition to achieving an efficacious therapeutic index [15].

Conventional wisdom concerning the etiology of adverse events related to HBOC stems primarily from nitric oxide (NO) scavenging at the endothelium, which paradoxically leads to vasoconstriction and decreased tissue oxygenation. Following unsuccessful phase II clinical trials for diaspirin cross-linked hemoglobin by Baxter, a number of developers sought hemoglobin derivatives modified to either reduce or compensate for NO binding. These strategies included: (1) increasing molecular weight of hemoglobin to prevent extravasation, (2) glutaraldehyde polymerization, (3) O-raffinose cross-linking human hemoglobin with unpolymerized tetramers, or (4) genetic modification. Unfortunately, advanced clinical trials of these modified hemoglobin did not yield promising results [16].

Analysis of these modified hemoglobin molecules noted another set of toxicities in addition to NO scavenging that would preclude safety and efficacy. These toxicities are related to pro-oxidant activity, a side effect not addressed by the aforementioned modifications. Cell-free hemoglobin, in the absence of the anti-oxidant enzymes found in the red blood cell, combines with oxygen to form free radicals such as superoxide, hydrogen peroxide, hydroxyl radical, and oxoferryl porphyrin among others [17]. Thus, the reduction in oxygen delivery resulting from hemoglobin's NO scavenging and vasoconstriction is further exacerbated by oxidative stress through a burst of superoxide generation from heme iron auto oxidation in the transfused HBOCs. In turn, cell-free hemoglobin without appropriate regulation adds to the inflammatory insult of ischemia and reperfusion, adding to the underlying pathology in the very clinical situations where a blood substitute would be most useful [18].

Strategies to address both the vasoconstrictive and pro-oxidant activity of cell-free hemoglobin have included: (1) co-polymerizing the HBOC with the antioxidant enzymes SOD and catalase, (2) pharmacological cross-linking the HBOC with ATP, adenosine and reduced glutathione, (3) hemoglobin modification to reduce P50, (4) S-Nitrosylating and PEGylating the HBOC, (5) adjunct therapies of nitric oxide inhalation or sodium nitrite. Detailed reviews of other HBOCs for use in military medicine were recently published [19–21]. The ideal HBOC would not only expand intravascular volume and increase oxygen delivery but do so in a manner that manages the oxidative insult of ischemia and reperfusion. A novel macromolecule developed by Carleton Hsia and co-workers is Polynitroxylated PEGylated Hemoglobin (PNPH; aka, SanFlow), a bovine hemoglobin stabilized by polyethylene glycol with added catalytic polynitroxyl groups that is poised to address both these problems and usher in a new paradigm in resuscitative medicine.

The Development of Polynitroxylated PEGylated Hemoglobin (SanFlow)

The precursor to PNPH was polynitroxylated albumin (PNA; aka VACNO or vascular albumin with caged nitric oxide) which has been shown to undergo reversible reduction and oxidation to function as a mimetic of superoxide dismutase and catalase. Interestingly, PNA is currently being developed for use in cancer following the demonstration that it has anti-metastatic effects in triple negative breast cancer animal models [22]. By adding polynitroxyl groups to pegylated hemoglobin, PNPH theoretically provides optimal resuscitation by attacking various dysregulations that are present in patients undergoing traumatic shock. PNPH (Fig. 23.1) is a ~ 8 nm nano particle with multifunctional catalytic anti-oxidative and hemodynamic colloid activities, has a single core Hb, which is shielded by an inner superoxide dismutase (SOD) mimetic nitroxide shell and an outer hydrated polyethylene glycol. PNPH's five structural and functional components are: (1) bovine hemoglobin as the protein center provides oxygen carrier capability but is carboxylated to provide thermo-stability and added anti-inflammatory activity, (2) the hydrated polyethylene glycol moieties of PNPH provide hyper-colloid properties important to stabilizing hemodynamics during hypotension and hypovolemia, (3) the nitroxide moieties of PNPH not only improve the safety of cell-free hemoglobin but also provide anti-oxidant/anti-inflammatory and neuroprotective activities, (4) desirable redox coupling of heme iron with the nitroxide is promoted in the stoichiometry, and (5) further redox coupling of the nitroxide/heme iron complex with endogenous plasma anti-oxidants such as ascorbate provides additional anti-oxidant activities [18]. The molecular focus on optimizing redox

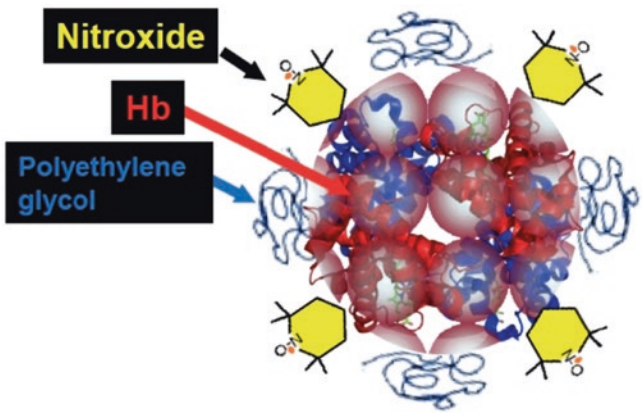


Fig. 23.1 Schematic of PNPH. Polynitroxide (yellow) and polyethylene glycol (blue) moieties decorate the Hb surface. Pegylation yields super colloid effects. The nitroxides mitigate reactive oxygen species, attenuating nitric oxide (NO) consumption, and oxidative injury to the microcirculation and the Hb molecule itself. PNPH has 12–14 nitroxides per Hb

potential, minimizing oxidative stress, and providing colloidal support for increase circulatory volume (instead of the traditional focus of oxygen delivery and NO supplementation) represents a paradigm shift in the development of an optimal blood substitute. The development of PNPH thus addresses key recommendations of the 2011 NIH/FDA/DoD Working Group on Oxygen Therapeutics (https://www.nhlbi.nih.gov/events/2011/oxygen-therapeutics). PNPH is currently classified by the military as a Technology Readiness Level 4 product. In PIND meetings with FDA, the FDA agreed that PNPH is more than an oxygen carrier, that it is a "therapeutic" with efficacies in multiple medical conditions such as TBI, hemorrhagic shock, stroke and sickle cell disease.

In order to support the paradigm shift that PNPH affords, this review will detail published data on the efficacy of PNPH in oxidative stress models of traumatic brain injury combined with hemorrhagic shock. The review of current published data will address the following novel therapeutic functions: (1) The new molecular design of PNPH greatly decreases the natural tendency of heme iron to auto oxidize and generate superoxide, thus preventing vasoconstriction and correcting inadequate blood flow, (2) PNPH is a potent neurovascular protectant through redox coupling of nitroxide moieties with heme, mimicking superoxide dismutase (SOD), (3) this redox coupling is augmented via interactions with plasma ascorbate, and (4) PNPH contains up to 14 nitroxyl groups shielded by a hydrated polyethylene glycol shell providing both stabilization and colloidal properties.

PNPH in Traumatic Brain Injury and Hemorrhagic Shock

A series of four preclinical papers have described PNPH as an efficacious, novel, anti-oxidative hyper-colloid neuroprotective small volume resuscitative fluid for TBI + HS when compared to standard therapy in a model simulating the prehospital setting [23–26]. Early work from Patrick Kochanek's lab at University of Pittsburgh [23] showed PNPH requires the least volume to restore and maintain mean arterial blood pressure (MAP) when compared to Lactated Ringer's (LR), (standard civilian therapy), or Hextend (HEX), (prior standard military therapy), and confers neuroprotection in a relevant mouse model of TBI + HS. Mice resuscitated with PNPH had fewer Fluoro-Jade C+ identified dying neurons in CA1 vs. HEX and LR. PNPH was also shown not only to be non-toxic but also to be neuroprotective against injury (glutamate/glycine exposure and neuronal stretch) in rat primary cortical neuron cultures ($p < 0.01$). PNPH also maintained cerebral oxygenation better that LR and HEX as measured by implanted direct oxygen electrodes [23]. A subsequent study from Patrick Kochanek's group [24] expounded on PNPH as a neuroprotective pre-hospital resuscitative therapeutic. Mice underwent controlled cortical impact followed by induced severe hemorrhagic shock where MAP of 25–27 mmHg was maintained for 35 minutes in the initial shock phase. Mice were then resuscitated with 20 mL/kg bolus of 4% PNPH or LR followed by 10 mL/kg boluses targeting MAP greater than 70 mmHg for 90 minutes in the prehospital phase. Afterwards, the blood shed was then re-infused in the hospital phase. Mice resuscitated with PNPH vs LR required less fluid (26.0 vs 167.0 mL/kg, $P < 0.001$) and had a higher MAP (79.4 vs 59.7 mmHg, $P < 0.001$). The PNPH-treated mice had lower peak intracranial pressures (ICP) (14.5 vs 19.7, $P = 0.002$) and required only 20 mL/kg of PNPH while LR-resuscitate mice required multiple crystalloid boluses [24].

In 2017, Patrick Kochanek's group went on to compare PNPH with (1) whole blood and (2) Lactated Ringer's in a small volume therapeutic TBI + HS resuscitation model, and found that PNPH eliminated the need for fluid administration, and reduced both intracranial pressure and brain edema [25]. Figure 23.2 shows the MAP time-course. Following an initial shock and bleeding to 35 min, MAP fell to 25 mm Hg. The target was to resuscitate to 70 mm Hg. The LR mice received 3 separate boluses every 5 min to complete resuscitation vs. whole blood and PNPH receiving a single bolus at the initiation of the resuscitation phase. Figure 23.2 shows that MAP restoration with PNPH exceeds 70 mmm Hg and is greater than with whole blood and, importantly, remains constant over the entire 90 min window. Whole blood MAP in contrast, following an initial peak above 60 mm Hg, subsequently declined throughout the 90 min observation period. With PNPH, there was no significant difference in MAP after resuscitation in the first 15 min compared with the final 15 min, but both the LR and whole blood groups experienced a progressive decline in MAP and had significantly lower MAPs ($P < 0.001$) in the final 15 min of the model. In dose response studies, mice were resuscitated with PNPH with doses between 2 and 100 mL/kg. PNPH-resuscitated mice had higher MAP and lower HR post-resuscitation versus LR ($p < 0.01$). PNPH-resuscitated mice, versus those resuscitated with blood or LR, also had higher pH and lower serum potassium ($p < 0.05$). PNPH was well tolerated and dramatically reduced fluid requirements across all dose ranges, even at the lowest doses of 2 or 5 mL/kg ($p < 0.001$). The two lowest doses of PNPH tested (2 mL/kg and 5 mL/kg) produced significant 1.5 and 3.2-fold reduction in total fluid requirements (Fig. 23.3). Resuscitation with PNPH proved to improve MAP, heart rate, intracranial pressure, reduced acidosis, reduced hyperkalemia, and was superior over whole blood or LR.

More recently, Raymond Koehlers' lab at Johns Hopkins reported in 2020 that PNPH afforded similar neuroprotective and vascular therapeutic benefits in a new guinea pig model

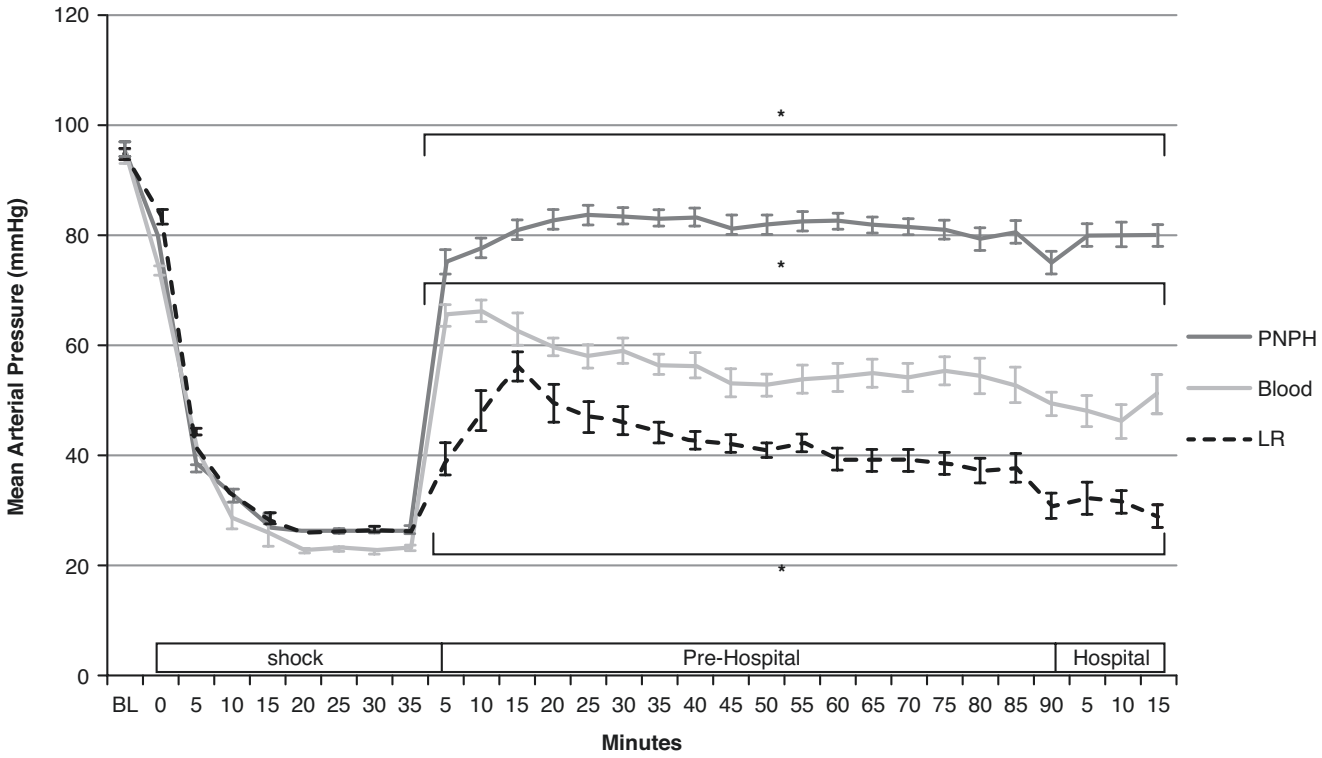


Fig. 23.2 Mean arterial blood pressure following traumatic brain injury accompanied by hemorrhagic shock in a "Pre-Hospital" + "Hospital" model in mice. MAPs were significantly different between all groups during the "Pre-Hospital" and "Hospital" phase (*P < 0.001). PNPH polynitroxylated pegylated hemoglobin, LR Lactated Ringers. PNPH, whole blood or LR were infused at the beginning of the Pre-Hospital phase. (From Brockman et al. 2017)

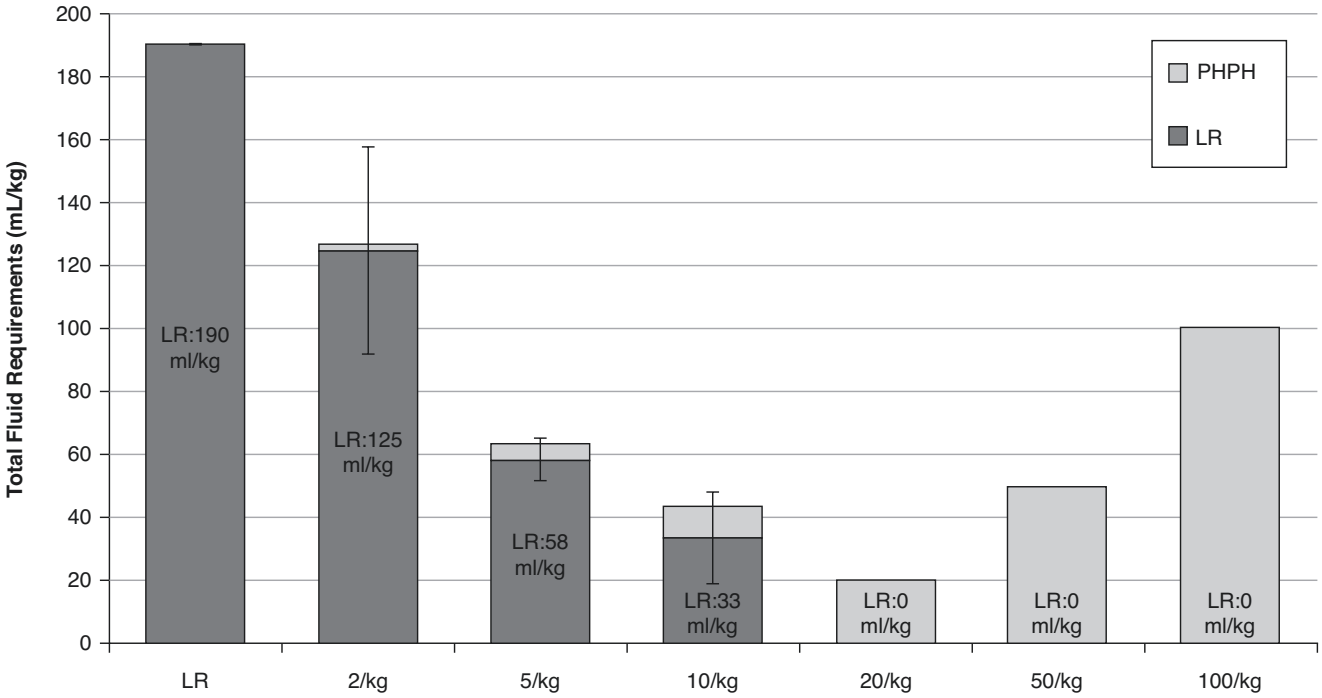


Fig. 23.3 Fluid requirements during PNPH dose response studies. Group designation shown on the x-axis and amount of LR needed in the y-axis. Mice resuscitated with LR required the most fluid versus all other PNPH dose groups ($p < 0.01$). PNPH resuscitations was associated with markedly reduced total fluid requirements versus LR at all dosages ($p < 0.01$). (From Brockman et al., 2017)

of TBI + HS [26]. The reason guinea pigs were studied was that guinea pigs, like humans, do not synthesize ascorbic acid. Ascorbic acid is ordinarily required for the reduction of metHb which is important because of the increased formation of highly reactive ferryl Hb and the oxidative damage that occurs when metHb and peroxide accumulate. This has been an important toxicity concern in using of Hb-based oxygen carriers in humans. Mice however do synthesize ascorbic acid and this in principle could have confounded earlier studies in mice. Testing of guinea pigs served to further test translatability to humans. It should also be mentioned that in the PNPH molecule, ascorbate participates in the redox cycling of the covalently labeled nitroxides on PNPH through an unusual redox-catalytic mechanism whereby reduction of H_2O_2 is achieved at the expense of reducing equivalents of ascorbate converted into reduced nitroxides [27]. TBI in the study by Seno et al. [26] was produced by controlled cortical impact and was followed by 20 mL/kg hemorrhage to a mean arterial pressure (MAP) of 40 mmHg. At 90 min, animals were resuscitated with 20 mL/kg lactated Ringer's solution or 10 mL/kg PNPH. Resuscitation with PNPH significantly augmented the early recovery of MAP after hemorrhagic shock by 10–18 mmHg. At 9 days of recovery, unbiased stereology analysis revealed that, compared to animals resuscitated with lactated Ringer's solution, those treated with PNPH had significantly more viable neurons in the hippocampus CA1 + 2 region (59 ± 10% versus 87 ± 18% of sham and naïve mean value) and in the dentate gyrus (70 ± 21% versus 96 ± 24%; n = 12 per group). The study evaluated recovery for up to 9 days, which is of high importance to combat casualty care, whereas earlier studies in mice evaluated resuscitation/ recovery for 90 minutes. The study provided detailed toxicological analyses supporting translatability of PNPH to humans. To further establish the translatability of PNPH to humans, PNPH in ongoing current work is being evaluated in two large animal models: (1) TBI + HS model in swine, (2) GLP toxicity studies in cynolmolgus monkeys, where studies to date indicate PNPH has a therapeutic index of >10 and is safe (unpublished data). Progression to clinical trials is expected within 12–18 months at time of writing.

Remote Pre-hospital Medicine Beyond the 'Golden Hour'

The term "Golden Hour" was coined by R Adams Cowley in 1957. Cowley was a former military surgeon who established the first state-wide medevac system for U.S. civilian medicine to promote the urgency of minimizing the time between trauma injury and definitive care in civilian medicine. In 2009, U.S. Secretary of Defense Robert M. Gates mandated a standard of 60 minutes or less for the evacuation time by helicopter of critically injured military personnel to a surgical team at a medical facility, which resulted in major declines in morbidity and mortality [28]. Today, however, the paradigm has shifted dramatically. The nature of military operations requires that medical capability be available remotely - in the next twenty-first century near peer battlefield, where communications and secure medevac will not be available, military medicine will need to maintain medical capabilities in isolated and widely distributed environments without the ability for resupply or the possibility of timely evacuation. Military mass casualty incidents also pose unique challenges, exemplified by the Navy case study by Day et al. [29] of the use of warm fresh whole blood (WFWB) in hemostatic resuscitations afloat. Despite the benefits of volume augmentation and oxygenation, blood products have some drawbacks including transfusion reaction, blood-borne illness, and immune dysregulation as well as unique logistical and practical constraints in the forward-deployed and contested air space environment.

Prolonged Field Care (PFC) has developed as a new paradigm and is currently the top priority capability gap across the US Army [30, 31]. PFC refers to medical care applied 'beyond doctrinal planning', and has much in common with medical care needed in wilderness environments and in disaster emergencies with limited resources, where evacuation of patients to needed medical infrastructure is greatly delayed or not possible. Space travel is now regarded as the ultimate example of PFC, and is of interest to NASA [32]. Closely related to PFC is the area of Remote Damage Control Resuscitation (RDCR) in the military and its current approved practices [33].

The gold standard of traumatic resuscitation in the forward deployed military setting remains stored whole blood or blood components, but logistical and practical constraints with storage, refrigeration, and maintenance make this a precious, limited resource. The use of fresh whole blood (FWB) or low-titer group O whole blood (LTOWB) are the top two methods of treatment in the remote setting, while crystalloids or Hextend are therapies of last resort and can worsen coagulopathy and bleeding [33].

If blood products are not immediately available, the Department of Defense Committee on Tactical Combat Casualty Care (TCCC) recommends a half-liter bolus of HEX for initial resuscitation of combat casualties. Colloids such as HEX are preferred over crystalloids for fluid resuscitation in the military according to the 2019 TCCC Guidelines. The initial benefit to HEX was the potential for significant volume expansion with a smaller volume, having obvious logistical and practical benefits in the austere medical environment with limited resources. The recommendation for HEX still holds despite a June 2013 US Food and Drug

Administration (FDA) warning for HEX increasing mortality and acute kidney injury after large volume civilian resuscitations [2].

What would be the ideal resuscitation agent for use in PFC/RDCR? The ability to adequately volume-resuscitate a patient is paramount, and the use of a low-volume, shelf-stable, colloid resuscitative fluid that would afford the benefit of providing oxygen-carrying capacity to the patient's organs would be transformative in resuscitative medicine. For use in austere environments and PFC, this ideal agent would need to be stable for prolonged periods without refrigeration. Currently, in far-forward deployed teams, there is difficulty storing and transporting blood components in a safe and effective manner. A key issue in the stability of any hemoglobin-based product is catabolism that releases heme and leads to iron toxicity - this is a key toxicity seen in cell-free hemoglobins and limits their usefulness. Other toxicities addressed by PNPH, including hemoglobin's NO scavenging, vasoconstriction, and pro-oxidant activity were reviewed previously [18].

In PNPH, the heme is CO ligated, the result of which is that the heme undergoes little or no autooxidation, but only as long as CO remains ligated. CO ligation is also a feature of other HBOCs such as Sanguinate [34] and is not unique to PNPH. Where PNPH is unique among all HBOCs is that it is the only agent that employs nitroxylation chemistry, which gives it unique benefits. Redox coupling due to nitroxide moieties stabilizes heme in PNPH; one demonstrated effect of this is loss of iron toxicity compared other cell-free hemoglobins in neuronal cell assays [20]. Nitroxylation also confers antioxidant properties, which is central to its unique efficacies seen in multiple medical conditions - PNPH is a superoxide dismutase (SOD) mimetic, catalase mimetic, and has NO scavenging activity [18]. With regard to shelf-life, PNPH is pasteurized at 70 °C during manufacturing and has shown stability for up to 2 years (unpublished data). Thus, PNPH may be unique among HBOCs in its suitability for use in the field for prolonged periods. We envision that PNPH (SanFlow) could be administered at Point of Injury by minimally trained personnel using technologies such as FAST1™ Intraosseous Infusion.

Summary

A growing body of preclinical evidence suggests that SanFlow represents a paradigm shift in HBOC development toward a new focus on correcting inadequate blood flow due to oxidative stress and attenuating ischemia/reperfusion/inflammation injury. Also, if this holds up in clinical trials, SanFlow appears likely to meet the FDA mandate for an HBOC with a meaningful increase in therapeutic index. The multifunctional, molecular mechanism of SanFlow suggests that it may have significant value in the forward deployed and pre-hospital setting. The unique logistical and practical constraints in the austere medical environment in addition to 2019 TCCC Guidelines HEX highlights a gap that SanFlow is poised to address. SanFlow should be further studied to better define the contribution to favorable outcome of each of the rich array of potential mechanistic effects of this multifunctional therapeutic. SanFlow has the potential to address major healthcare concerns and unmet medical needs in military and resuscitative medicine, in both pre-hospital and in-hospital settings.

Key Points

- PNPH (SanFlow) as a resuscitation agent is an effective alternative to whole blood and provides better improvement in MAP and heart rate in TBI + HS animal models, plus decreases intracranial pressure/edema, acidosis and hyperkalemia.
- PNPH is effective at low volumes and addresses the dilutional coagulopathy seen with other resuscitation agents.
- As a result of its nitroxylation chemistry, PNPH does not show the rapid irreversible conversion to methemoglobin found in earlier HBOCs and the nitric oxide scavenging activity of cell-free hemoglobin is inhibited.
- PNPH provides organ protection in the brain, and addresses cardiovascular and renal dysfunction toxicities seen with prior HBOCs.
- PNPH is stable, does not require refrigeration and would be suitable for use in the field in military deployments and disaster emergencies.

Conflict of Interest CJC Hsia holds shares in AntiRadical Therapeutics, which holds the license for polynitroxylated PEGlyated hemoglobin (PNPH; aka SanFlow); CJC Hsia and BJ Soltys are board members at AntiRadical Therapeutics; CJC Hsia is recipient of SBIR contracts from the Department of Defense to study the potential use of SanFlow in combat casualty care (US Army Medical Research and Materiel Command Contracts W81XWH-17-C-0223 and W81XWH19C0022). The views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

References

1. Cap AP, Pidcoke HF, DePasquale M, DePasquale M, Rappold JF, Glassberg E, et al. Blood far forward: time to get moving! J Trauma Acute Care Surg 2015;78(6 Suppl 1):S2–S6. doi: https://doi.org/10.1097/TA.0000000000000626 . PMID: 26002259.
2. Butler FK, Holcomb JB, Schreiber MA, Kotwal RS, Jenkins DA, Champion HR, et al. Fluid resuscitation for hemorrhagic shock in tactical combat casualty care: TCCC guidelines change 14-01–2 June 2014. J Spec Oper Med. 2014;14(3):13–38. PMID: 25344706
3. Shackelford SA, Del Junco DJ, Powell-Dunford N, Mazuchowski EL, Howard JT, Kotwal RS, et al. Association of prehospital blood product transfusion during medical evacuation of combat casualties in Afghanistan with acute and 30-day survival. JAMA. 2017;318(16):1581–91. https://doi.org/10.1001/jama.2017.15097. PMID: 29067429.
4. Learoyd P. The history of blood transfusion prior to the 20th century–part 1. Transfus Med. 2012;22(5):308–14. https://doi.org/10.1111/j.1365-3148.2012.01180.x. PMID: 22994447.
5. COL Andrew P Cap MU, LTC Andrew Beckett MC, MAJ Avi Benov MI, et al. Whole blood transfusion (CPG ID: 21). In:15 May 2018: https://jts.amedd.army.mil/assets/docs/cpgs/JTS_Clinical_Practice_Guidelines_(CPGs)/Whole_Blood_Transfusion_15_May_2018_ID21.pdf. Accessed 12 Jan 2020.
6. Simmons RL, Collins JA, Heisterkamp CA, Mills DE, Andren R, Phillips LL. Coagulation disorders in combat casualties. I. Acute changes after wounding. II. Effects of massive transfusion. 3. Post-resuscitative changes. Ann Surg. 1969;169(4):455–82. https://doi.org/10.1097/00000658-196904000-00001. PMID: 5774736.
7. Ferenz KB, Steinbicker AU. Artificial oxygen carriers-past, present, and future-a review of the most innovative and clinically relevant concepts. J Pharmacol Exp Ther. 2019;369(2):300–10. https://doi.org/10.1124/jpet.118.254664. Epub 2019 Mar 5. PMID: 30837280.
8. Keipert PE, Faithfull NS, Roth DJ, Bradley JD, Batra S, Jochelson P, Flaim KE. Supporting tissue oxygenation during acute surgical bleeding using a perfluorochemical-based oxygen carrier. Adv Exp Med Biol. 1996;388:603–9. https://doi.org/10.1007/978-1-4613-0333-6_77. PMID: 8798865.
9. Lowe KC. Engineering blood: synthetic substitutes from fluorinated compounds. Tissue Eng. 2003;9(3):389–99. https://doi.org/10.1089/107632703322066570. PMID: 12857407
10. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature. 2007;448(7151):313–7. https://doi.org/10.1038/nature05934. Epub 2007 Jun 6. PMID: 17554338.
11. Giarratana MC, Rouard H, Dumont A, Kiger L, Safeukui I, Le Pennec PY, et al. Proof of principle for transfusion of in vitro-generated red blood cells. Blood. 2011;118(19):5071–9. https://doi.org/10.1182/blood-2011-06-362038. Epub 2011 Sep 1. PMID: 21885599.
12. Amberson WR, Mulder AG, Steggerda FR, Flexner J, Pankratz DS. Mammalian life without red blood corpuscles. Science. 1933;78(2014):106–7. https://doi.org/10.1126/science.78.2014.106. PMID: 17732672
13. Amberson WR, Jennings JJ, Rhode CM. Clinical experience with hemoglobin-saline solutions. J Appl Physiol. 1949;1(7):469–89. https://doi.org/10.1152/jappl.1949.1.7.469. PMID: 18104040.
14. Stern SA. Low-volume fluid resuscitation for presumed hemorrhagic shock: helpful or harmful? Curr Opin Crit Care. 2001;7(6):422–30. https://doi.org/10.1097/00075198-200112000-00009. PMID: 11805545.
15. Silverman TA, Weiskopf RB, Planning Committee and the Speakers. Hemoglobin-based oxygen carriers: current status and future directions. Anesthesiology. 2009;111(5):946–63. https://doi.org/10.1097/ALN.0b013e3181ba3c2c. PMID: 19858869.
16. Sloan EP. The clinical trials of diaspirin cross-linked hemoglobin (DCLHb) in severe traumatic hemorrhagic shock: the tale of two continents. Intensive Care Med. 2003;29(3):347–9. https://doi.org/10.1007/s00134-003-1637-y. PMID: 12710459.
17. Gould SA, Moore EE, Hoyt DB, Ness PM, Norris EJ, Carson JL, Hides GA, Freeman IH, DeWoskin R, Moss GS. The life-sustaining capacity of human polymerized hemoglobin when red cells might be unavailable. J Am Coll Surg 2002;195(4):445–452.; discussion 452–5. https://doi.org/10.1016/s1072-7515(02)01335-2. PMID: 12375748.
18. Hsia CJ, Ma L. A hemoglobin-based multifunctional therapeutic: polynitroxylated pegylated hemoglobin. Artif Organs. 2012;36(2):215–20. https://doi.org/10.1111/j.1525--1594.2011.01307.x. Epub 2011 Sep 28. PMID: 21955160.
19. Bialas C, Moser C, Sims CA. Artificial oxygen carriers and red blood cell substitutes: a historic overview and recent developments toward military and clinical relevance. J Trauma Acute Care Surg. 2019;87(1S Suppl 1):S48–58. https://doi.org/10.1097/TA.0000000000002250. PMID: 31246907
20. Gupta AS. 2017 military supplement: hemoglobin-based oxygen carriers. Shock. 2017;52:1. https://doi.org/10.1097/SHK.0000000000001009. PMID: 31513123
21. Edwards TH, Hoareau GL. Fluids of the future. Front Vet Sci. 2021;7:623227. https://doi.org/10.3389/fvets.2020.623227. PMID: 33553287.
22. Messerli SM, Schaefer AM, Zhuang Y, Soltys BJ, Keime N, Jin J, Ma L, Hsia CJC, Miskimins WK. Use of antimetastatic SOD3-mimetic albumin as a primer in triple negative breast cancer. J Oncol. 2019;2019:3253696. https://doi.org/10.1155/2019/3253696. PMID: 30941174; PMCID: PMC6420975.
23. Shellington DK, Du L, Wu X, Exo J, Vagni V, Ma L, Janesko-Feldman K, et al. Polynitroxylated pegylated hemoglobin: a novel neuroprotective hemoglobin for acute volume-limited fluid resuscitation after combined traumatic brain injury and hemorrhagic hypotension in mice. Crit Care Med. 2011;39(3):494–505. https://doi.org/10.1097/CCM.0b013e318206b1fa. PMID: 21169820.
24. Brockman EC, Bayır H, Blasiole B, Shein SL, Fink EL, Dixon C, et al. Polynitroxylated-pegylated hemoglobin attenuates fluid requirements and brain edema in combined traumatic brain injury plus hemorrhagic shock in mice. J Cereb Blood Flow Metab. 2013;33(9):1457–64. https://doi.org/10.1038/jcbfm.2013.104. Epub 2013 Jun 26. PMID: 23801241.
25. Brockman EC, Jackson TC, Dixon CE, Bayır H, Clark RS, Vagni V, et al. Polynitroxylated pegylated hemoglobin-a novel, small volume therapeutic for traumatic brain injury resuscitation: comparison to whole blood and dose response evaluation. J Neurotrauma. 2017;34(7):1337–50. https://doi.org/10.1089/neu.2016.4656. Epub 2017 Jan 13. PMID: 27869558.
26. Seno S, Wang J, Cao S, Saraswati M, Park S, Simoni J, et al. Resuscitation with macromolecular superoxide dismutase/catalase mimetic polynitroxylated PEGylated hemoglobin offers neuroprotection in guinea pigs after traumatic brain injury combined with hemorrhage shock. BMC Neurosci. 2020;21(1):22. https://doi.org/10.1186/s12868-020-00571-7. PMID: 32404052.
27. Stoyanovsky DA, Kapralov A, Huang Z, Maeda A, Osipov A, Hsia CJ, et al. Unusual peroxidase activity of polynitroxylated pegylated hemoglobin: elimination of H(2)O(2) coupled with intramolecular oxidation of nitroxides. Biochem Biophys Res Commun. 2010;399(2):139–43. https://doi.org/10.1016/j.bbrc.2010.07.030. Epub 2010 Jul 17. PMID: 20643098.
28. Kotwal RS, Howard JT, Orman JA, Tarpey BW, Bailey JA, Champion HR, Mabry RL, Holcomb JB, Gross KR. The effect of a Golden hour policy on the morbidity and mortality of combat casualties. JAMA Surg. 2016;151(1):15–24. https://doi.org/10.1001/jamasurg.2015.3104. PMID: 26422778.

29. Day WG, Cooper E, Phung K, Miller B, DuBose J, Lin AH. Prolonged stabilization during a mass casualty incident at sea in the era of distributed maritime operations. Mil Med. 2020;185(11–12):2192–7. https://doi.org/10.1093/milmed/usaa147. PMID: 32870304.
30. Keenan S, Riesberg JC. Prolonged field care: beyond the "Golden Hour". Wilderness Environ Med. 2017;28(2S):S135–9. https://doi.org/10.1016/j.wem.2017.02.001. PMID: 28601206.
31. Dolan CP, Valerio MS, Lee Childers W, Goldman SM, Dearth CL. Prolonged field care for traumatic extremity injuries: defining a role for biologically focused technologies. NPJ Regen Med. 2021;6(1):6. https://doi.org/10.1038/s41536-020-00117-9. PMID: 33542235.
32. Schlotman TE, Lehnhardt KR, Abercromby AF, Easter BD, Downs ME, Akers LTCKS, Convertino VA. Bridging the gap between military prolonged field care monitoring and exploration spaceflight: the compensatory reserve. NPJ Microgravity. 2019;5:29. https://doi.org/10.1038/s41526-019-0089-9. PMID: 31815179; PMCID.
33. Fisher AD, Washburn G, Powell D, Callaway DW, Miles EA, Brown J, Dituro P. Damage Control Resuscitation (DCR) in Prolonged Field Care (PFC) (CPG ID:73), Joint trauma system clinical practice guideline (JTS CPG. https://jts.amedd.army.mil/assets/docs/cpgs/Damage_Control_Resuscitation_PFC_01_Oct_2018_ID73.pdf. Accessed 1 May 2021.
34. Alayash AI. Mechanisms of Toxicity and Modulation of Hemoglobin-based Oxygen Carriers. Shock. 2019;52(1S Suppl 1):41–9. https://doi.org/10.1097/SHK.0000000000001044. PMID: 29112106.

Erythromer (EM), a Nanoscale Bio-Synthetic Artificial Red Cell

24

Nivesh Mittal, Stephen Rogers, Shannon Dougherty, Qihong Wang, Parikshit Moitra, Mary Brummet, Elyse M. Cornett, Alan D. Kaye, Sahar Shekoohi, Paul Buehler, Philip Spinella, Dipanjan Pan, and Allan Doctor

Introduction

Unmet Need There is need for an artificial oxygen (O_2) carrier for use when banked blood is unavailable or undesirable. To address this need, we developed **ErythroMer (EM)**, a first-in-class, bio-synthetic, nano-cyte blood substitute. EM is a deformable, hybrid peptidic-lipid nanoparticle that incorporates high per particle payloads of hemoglobin (Hb) in a fashion that both (a) confers context-responsive control of O2 capture and release and (b) limits adverse reaction between Hb and nitric oxide (NO). Our 'artificial cell' design has yielded a prototype that emulates key RBC physiology and represents a potentially disruptive introduction into Transfusion Medicine. To date, efforts to develop Hb-based oxygen carriers (HBOCs) have failed, because of design flaws which do not preserve physiologic interactions of Hb: (1) HBOCs capture O_2 in lungs, but do not release O_2 effectively to tissue, and (2) HBOCs trap nitric oxide (NO), causing vasoconstriction. The EM design (Fig. 24.1) surmounts these weaknesses by: (1) encapsulating Hb in a nanoparticle with novel (toroid) geometry (optimized surface area to volume ratio), (2) controlling O_2 capture/release with a novel shuttle for a small-molecule designed to lower Hb O_2 affinity (RSR-13, efaproxiral [1–6]), (3) attenuating NO uptake through shell properties, and (4) retarding metHb formation by co-packaging a reduction system. Moreover, EM is designed for sterile lyophilization and so, is amenable to facile reconstitution after extended, ambient dry storage. EM offers a pragmatic approach to a complex

N. Mittal · S. Dougherty
KaloCyte, Inc., Baltimore, MA, USA

S. Rogers · Q. Wang · M. Brummet
Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

Center for Blood Oxygen Transport and Hemostasis (CBOTH), University of Maryland School of Medicine, Baltimore, MD, USA

P. Moitra
Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

Center for Blood Oxygen Transport and Hemostasis (CBOTH), University of Maryland School of Medicine, Baltimore, MD, USA

Department of Diagnostic Radiology, University of Maryland School of Medicine, Baltimore, MD, USA

E. M. Cornett · S. Shekoohi
Department of Anesthesiology, LSU Health Sciences Center, Shreveport, Shreveport, LA, USA

A. D. Kaye
Department of Anesthesiology and Department of Pharmacology, Toxicology, and Neurosciences, LSU School of Medicine, Shreveport, LA, USA

Department of Anesthesiology and Pharmacology, LSU School of Medicine, New Orleans, LA, USA

Department of Anesthesiology and Pharmacology, Tulane School of Medicine, New Orleans, LA, USA

P. Buehler
Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

Center for Blood Oxygen Transport and Hemostasis (CBOTH), University of Maryland School of Medicine, Baltimore, MD, USA

Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, USA

P. Spinella
Department of Pediatric Critical Care, Washington University in St Louis, St. Louis, MO, USA

D. Pan
Department of Chemical, Biochemical and Environmental Engineering and Department of Computer Science and Electrical Engineering, University of Maryland Baltimore County, Baltimore, MD, USA

A. Doctor (✉)
Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA

Center for Blood Oxygen Transport and Hemostasis (CBOTH), University of Maryland School of Medicine, Baltimore, MD, USA

University of Maryland School of Medicine, Health Sciences Facility (HSF) III, Baltimore, MD, USA
e-mail: adoctor@som.umaryland.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_24

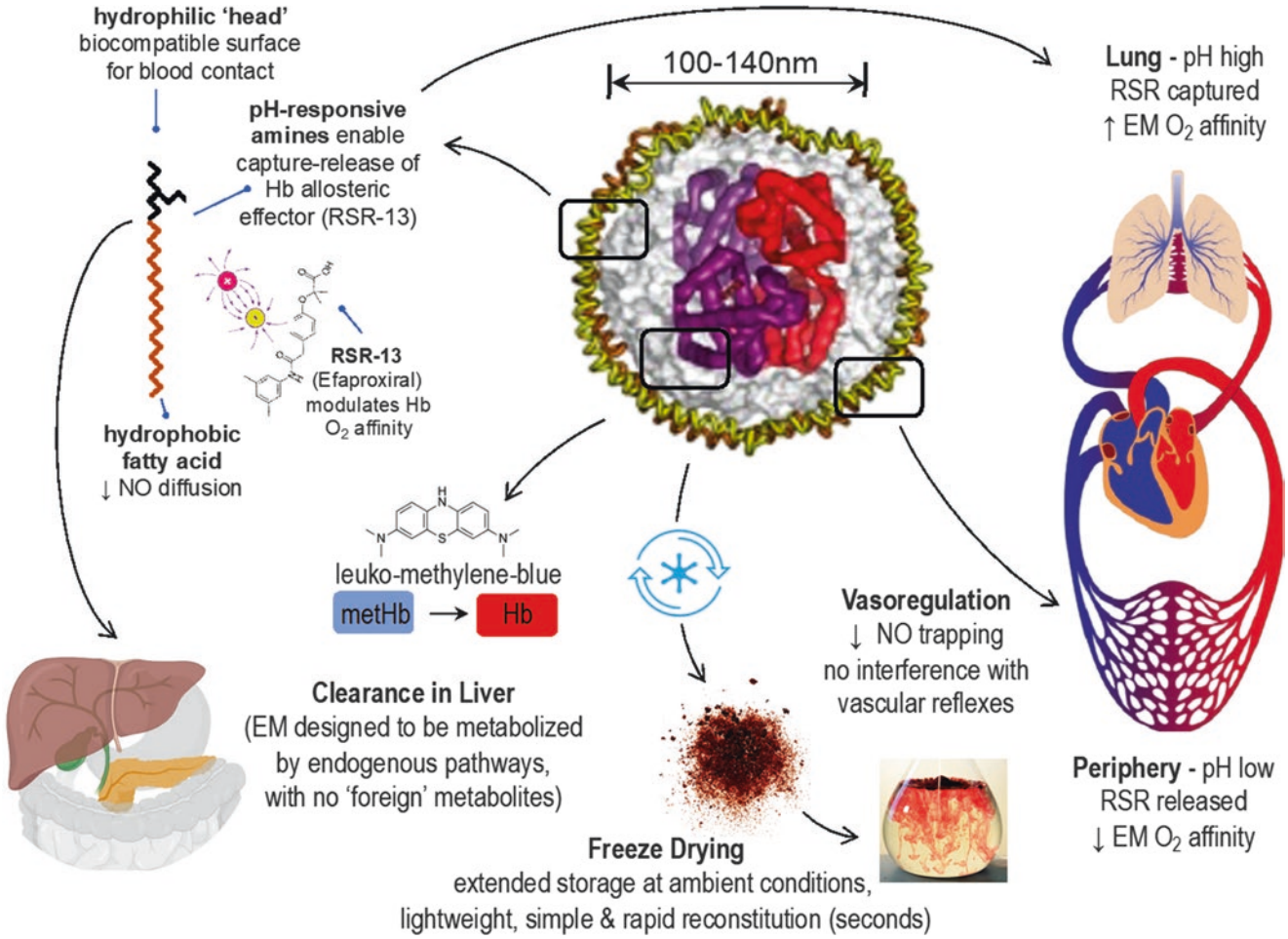


Fig. 24.1 ErythroMer Bioinspired Design: The novel amphiphilic peptidic-lipid precursor comprises: pH responsive groups that control availability of the allosteric effector (RSR13), enabling context-responsive control of O_2 binding and a negatively charged 'head,' facilitating biocompatibility of the exofascial surface. The shell is designed to retard NO consumption by Hb that is sequestered as within-particle payload. The precursor construct mimics endogenous biomolecules and is subject to enzymatic digestion and complete degradation *in vivo* to end-products identical that of 'natural' peptides and lipids

need and is designed for cost-effective production at scale. Our prototype has passed rigorous initial *ex vivo* and *in vivo* "proof of concept" testing.

Historical Context

Two major approaches have been pursued to date: perfluorocarbon emulsions (PFCs) and modified Hb agents (HBOCs) [7–9]. Despite promising preclinical data, neither has been successfully translated to clinical use [10, 11], possibly because designs do not emulate normal RBC physiology – resulting in interactions with NO and O_2 that disrupt homeostatic controls (particularly, controls matching vascular tone to tissue metabolism, e.g. hypoxic vasodilation (HVD) [12–14]. Normally, O_2 ligation by Hb initiates conformational shifts that result in a co-operative increase in affinity for additional O_2 [15, 16]. Consequently, Hb loads/unloads significant O_2, even across shallow O_2 gradients. This effect is amplified in RBCs by interaction between Hb and allosteric effectors (H^+, Cl^-, CO_2, and 2,3-DPG) [17]. However, when de-compartmentalized from the intra-erythrocytic milieu, (1) Hb loses allosteric controls and exhibits abnormally high O_2 affinity and (2) globin-chain crosslinking (required to stabilize HBOC tetramers) interferes with normal cooperativity. Both changes impair O_2 delivery. Moreover, lacking a membrane barrier to NO diffusion, decompartmentalized Hb disturbs vasoregulation due to avid intravascular NO trapping/consumption; this is further worsened by the propensity for free tetrameric Hb products to extravasate, binding NO in vessel walls, as well [18–22]. Such impaired vasoregulation by blood substitutes is a critical problem; because this effect reduces microcirculatory blood flow, O_2 delivery even by native RBCs is prevented [23] (and moreso, to hypoxic tissue, by impairing HVD). Chemically modified cell-free Hbs (designed to blunt this effect) have also suffered poor translation, due to an unfavorable risk-benefit profile: an HBOC meta-analysis demonstrated a significant increase in hyper-

tension, myocardial damage and mortality in surgical patients [24]; this finding has been recently reviewed, experimentally examined and reanalyzed, corroborating the concern [25–32]. Alternatively, perfluorocarbon-based O_2 carriers exhibit few side effects. However, for any given pO_2, Hb binds significantly more O_2 than can be dissolved in PFCs, and in contrast to the Hb sigmoidal binding/release curve, PFCs demonstrate a flat O_2 solubility curve. As a result, most of the O_2 carried by PFCs is prematurely released [33, 34], limiting tissue delivery [35]. Finally, neither PFCs nor most HBOCs can be lyophilized for prolonged storage at ambient conditions. At this time, the majority of products under active development are RBC-imitating vesicles or nanoparticles; these continue to struggle with: (1) complement activation by liposomal shells, (2) static O_2 affinity, (3) NO trapping, (4) complex metHb reduction systems, (5) inadequate shelf-life stability and (6) designs not amenable to lyophilization [36–42].

Bioinspired Design and Preclinical Results

Design Strategy

EM [43] has been designed with five major innovations (Table 24.1), including unique shape, morphology and biocompatibility, resembling RBCs. EM is a first-in-class, biosynthetic, nanocyte that modulates O_2 affinity to context during circulation (lungs ↔ tissue), slows NO trapping while permitting O_2 diffusion, recycles oxidized Hb via simple reduction and allows lyophilization, facile reconstitution, and extended shelf life (Fig. 24.2). EM is a self-assembled, deformable, peptidic-lipid amphiphile-based nanoparticle that incorporates a high hemoglobin (Hb) payload. The EM shell is composed of an amphiphilic precursor with pH-responsive peptidic groups that serve as 'wetware' by linking availability of the allosteric effector RSR13 [44–49] to biochemical cues of perfusion sufficiency. This design recapitulates context-responsive control of O_2 binding in RBCs. Also, the construct's phosphatidylcholine 'head' group facilitates biocompatibility of the exofacial surface, mimicking surface features of endogenous biomolecules and is subject to enzymatic digestion and degradation *in vivo* to end-products identical to that of 'natural' amino acids, lipids, and endogenous biomolecules.

Table 24.1 Analysis of 'bio-inspired' design solutions to challenges to HBOC efficacy, safety and pragmatic translation to clinical use

	HBOC flaw	EM solution
Oxygen (O_2) transport from lungs to tissue		
Design issue	O_2 affinity is fixed, Not context-responsive	pH-responsive RSR-13 shuttle
Consequence	Adequate O_2 capture (lungs), Poor O_2 release (tissue)	O_2 affinity shifts during transit (lungs ↔ tissue), links O_2 release to tissue need
Interference with normal regulation of blood vessel caliber		
Design issue	Traps the endogenous Vasodilator NO	Novel, 'tuneable' Peptidic-lipid shell
Consequence	Vasoconstriction, Tissue ischemia	Permits O_2 diffusion, Retards NO trapping, Vascular tone unaltered
Maintenance of hemoglobin (Hb) function during circulation		
Design issue	Hb auto-oxidizes, Generating metHb	Leuko-MB packaged In particle payload
Consequence	Limits Effective circulation time	Simple reduction system Re-generates oxidized Hb
Storage and ease of use		
Design issue	Incompatible With dry storage	Particle can be lyophilized
Consequence	Limits shelf life And versatility	Extends shelf life, facile use

Fabrication and Morphology

EM represents a new class of Hb-encapsulated, vascular constrained nanoparticle that is formulated by self-assembly of amphiphilic peptidic-lipid hybrid precursor. EMV2 (final formulation) has been designed with biocompatible novel lipid- amphiphile across the surface, producing a net negative zeta potential, excellent payload retention and differential gas diffusivity. Moreover, unlike phospholipid bilayers in liposomal-based HBOCs, EM has a tunable membrane offering greater integrity due to counterionic Hb and precursor interaction and pH responsive electrostatic interaction with a small molecule allosteric effector (RSR13). EM can be classified as a hybrid-vesicle resulting from the combined self-assembly of amphiphilic lipid mixtures- into an advanced vesicular structure. To afford such a design, the different parameters controlling both self-assembly and membrane structure have been optimized. The lipid-amphiphiles used in EM are mostly natural components of the cell membrane. Optimization studies revealed that hybrid vesicular structures can be obtained according to the molar composition and thermodynamic phase of the phospholipids and precursor mixture. In a typical procedure, EM vesicles are assembled in three steps. First, a thin film is prepared from the mixture of the novel hybrid peptidic-lipid precursor molecule (KC-1003), cholesterol and PEG_{2000}-PE by dissolving in anhydrous chloroform, which is then slowly evaporated under reduced pressure by rotary evaporator and dried. Hb is transferred to the dried film, RSR-13 (5:1, RSR-13: Hb), cryoprotectant (0.1% PEG_{400}) and lyoprotectant (1% Trehalose) are added to the Hb before transferring to the thin film, which is then immediately subjected to reverse phase solvent evaporation for particle self-assembly via microfluidization, after which contents are loaded into a

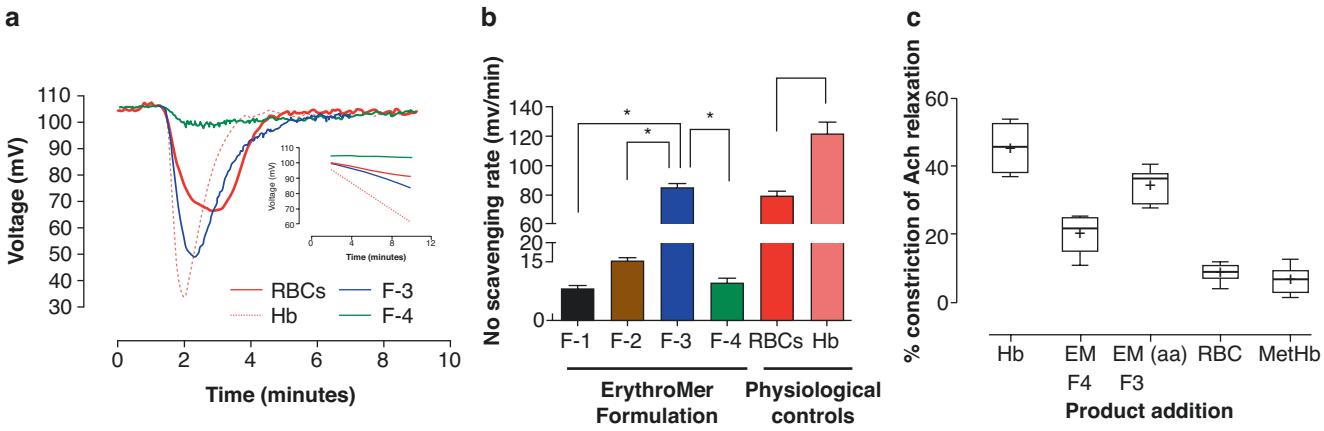


Fig. 24.2 NO sequestration. We adapted a validated NO consumption assay, linking a special reaction cell to our chemiluminescent NO analyzer. Spermine NONOate is injected into the reaction cell purged with Argon, carrying evolved NO to the analyzer and establishing baseline signal (e.g., voltage from photoelectric cell). Intact RBCs, free Hb, or EM (all equimolar for Hb) are then injected into the reaction cell; a decrease in signal is observed, which represents NO consumption by heme. The rate and total consumption for samples is determined as AUC. We examined this property in EM prototypes as a function of [Hb] payload and membrane features to achieve minimal NO sequestration rates. (**a**) Raw traces and (**b**) mean initial NO scavenging rates from the four EM preps, and from RBCs, free Hb, and the least & most promising EM preps (F3 & F4), all equimolar for Hb. Inset: Initial 10s. Free Hb sequesters NO more than RBCs; all EM formulations sequester NO less than RBCs. Initial scavenging rate; all EM preps differ from RBC & Hb excepting F3 (equivalent). *$p < 0.05$; $n = 5$; ANOVA. The physiologic relevance of these biochemical findings are affirmed by similar results from a vascular ring array (**c**), in which the degree to which exogenously added RBCs, freeHb, or EM abrogates an Ach-induced vasodilation (an NO mediated response)

Table 24.2 ErythroMer release specifications

	Assay	Requirement	Instrument
Wet (pre-lyophilized)	P50 (O_2 affinity)	(pH: P50) 7.2: 30 Torr 7.4: 22 Torr 7.6: 19 Torr	HemeOx analyzer
	Hill n (cooperativity)	2.5	HemeOx analyzer
	Hb ligation (HbCO)	> 98%	Co-oximetry
	NO trapping	Equal to fresh RBC	Chemi-luminescence
	Vasoactivity	Equal to fresh RBC	Vascular Ring Array
	Particle size	180 nm	Dynamic light scattering (DLS)
	Polydispersity	0.25	DLS
	Zeta potential	−20 mV	Electrophoretic
	Hb payload retention	> 98%	ICP-MS Encapsulation assay
	pH	7.4	pH meter
	Endotoxin	< 0.1 EU/mL	Gel clot PLUS SPI chromogenic
Dry	% heme	0.3 M	Energy Dispersive XRay
	% mass polymer	80%	Elemental analysis
	% mass cryoprotectant	0.1–0.5 M	GC/LC-MS
	Endotoxin	< 0.125 EU/mL	See above

Tangential Flow Filtration (TFF) System for cleanup and the formulation is checked against release specifications (Table 24.2). At the end of cleanup, supplemental cryo- and lyo-protectants are added to the suspension fluid (0.1% and 4% respectively) and EM is then freeze dried in vials using a shelf lyophilizer.

Self-assembly of the shell components in the presence of purified Hb results in a bilayer structure (hydrated state size measurements with dynamic light scattering = 120 ± 5 nm (w/o Hb) and 130 ± 10 nm (with Hb)); with polydispersity: 0.15 ± 0.01 and 0.21 ± 0.01, respectively, and zeta potential: −34 ± 4 mV), as described [50]. In the anhydrous state, tapping mode atomic force microscopy (AFM) and transmission electron microscopy (TEM) revealed slightly diminished particle height (H_{av} = 100 ± 20 nm) and diameter (D_{av} = 87 ± 12 nm). Preliminary evaluation of EM shelf-life stability (3 m, 25 °C) is established for both lyophilized and non-lyophilized particles. EM was tested for variation using particle diameter (solution state dynamic light scattering, DLS), zeta potential and polydispersity changes as initial criteria. Nominal change (<10%) in hydrodynamic diameter, zeta potential, or polydispersity was observed.

Inhibition of Hb Auto-oxidation

To address occasional superoxide (O_2^-) release and heme auto-oxidation (metHb formation) [51], RBCs recycle metHb by an NADH-powered enzymatic reduction system [52, 53]. As such, metHb accrual during circulatory transit is inevitable for any HBOC. Since oxidized hemes cannot bind O_2, this process progressively diminishes O_2 transport, limiting functional circulation time, possibly

prior to physical clearance [9, 54–57]. Complex approaches to this problem have been introduced [58–62], but challenge translation and realistic scale-up. EM uniquely incorporates leucomethylene blue (LMB) to inhibit metHb formation during formulation, storage, and use. LMB is the physiologically activated agent that reduces heme in treatment of methemoglobinemia [63–66]. It is FDA-approved and encapsulated in the EM payload.

Control of NO Sequestration

Vasoconstriction arising from NO sequestration is a challenge to HBOC safety [10, 67–69], particularly in the setting of endothelial dysfunction and physiologic stress [69–71]. Attempts to mitigate this with co-administered NO donors [72–74] or NO-donor adduction to Hb [75–78] have failed, largely because these approaches disrupt endogenous control of NO distribution and flux that are central to the physiologic vascular reflexes that dynamically govern blood flow distribution during shock/resuscitation [35, 79–82]. This endogenous physiologic control system involves context-responsive NO-based signaling between RBCs and endothelium that links vascular tone (and blood flow) to O_2 availability in the lung [83–86] and to O_2 consumption in the periphery [14, 87–91]. Under normal physiological conditions, RBCs *either* sequester *or* export NO, as they traverse ascending (lung) or descending (periphery) O_2 gradients, respectively [14, 92]. In normal RBCs, NO sequestration by Hb is abrogated by membrane properties, cell morphology, and rheology – which limit interaction between heme-Fe and endothelial-derived NO [14, 93–99]. EM emulates this physiology by similarly enveloping Hb in the nanoparticle shell; our findings indicate that EM does not sequester NO, permitting unbiased evolution of the vascular reflexes that dynamically link regional blood flow to tissue need, a critical advance over prior HBOCs.

Intelligent Control System (e.g., 'Wetware') for Modulation of EM O_2 Affinity

EM design enables *true physiologic linkage* between O_2 affinity and tissue respiration *via* a novel shuttle-reservoir of a small molecule Hb heterotropic effector. Conventional HBOC or PFC emulsions increase arterial O_2 content; however, *release* of O_2 in synchrony with endogenous physiologic control systems is difficult to emulate effectively *in vivo*, (i.e., along a physiological O_2 dissociation curve), due to lack of normal allosteric control of O_2 affinity (for Hb, expressed as P_{50}, the P_{O2} at which Hb O_2 saturation ($SHbO_2$) is 50%). In RBCs, P_{50} is modulated along two timescales, subacute (whole-(RBC)-population adaptation of all circulating RBCs to overall hypoxia or anemia) and real-time, acute (within-(RBC)-supbpopulation adjustment of RBCs P_{50} to moment-specific conditions of individual vascular beds). Subacute P_{50} adaptation is regulated by *2,3*-Diphosphoglycerate (2,3-DPG), a product of O_2-responsive glycolytic flux in RBCs and the major heterotropic effector modulating P_{50}. 2,3-DPG stabilizes deoxyHb conformation, diminishing O_2 affinity (higher P_{50}). During hypoxia, RBC 2,3-DPG rises slowly, over days, increasing O_2 offloading. Acute (real-time) P_{50} adaptation in RBC subpopulations is regulated by H^+, Cl^- and CO_2 which lower O_2 binding affinity (e.g. raise P_{50}) in proportion to their levels in RBCs. This phenomenon, called the Bohr effect, arises from interactions among the above heterotropic effectors bound to different sites on hemoglobin, all of which serve to stabilize the low energy, low affinity, T-state Hb conformation. The Bohr effect is achieved by complex interactions between carbonic anhydrase (CA) and the anion exchange protein 1 (AE1) (also known as the band 3 (B3) membrane protein) which link P_{50} to context (by 'transducing' the immediately proximal biochemical milieu) during circulatory transit (low P_{50} in lung, high in periphery). In sum, this physiology vastly improves O_2 transport efficiency by enhancing O_2 capture in the lung and release to tissue and does so in proportion to perfusion sufficiency (in the setting of impaired perfusion, acidosis and hypercapnea improve O_2 release). This relationship is highly relevant to transfusion efficacy, in which O_2 content is depressed by anemia PLUS_SPI /– hypoxemia. As such, facile P_{50} control is essential to robust HBOC design, affording ability to tailor high or low P_{50} formulations to clinical context. Unlike other HBOCs, EM employs an innovative shuttle-reservoir to modulate interaction between Hb and RSR13, a small molecule allosteric effector of Hb ~ O_2 affinity. Specifically, the pH-responsive nature of the amines in the novel peptidic-lipid amphile component of the EM shell (pKa values of 1°, 2° and 3° amines are approximately 9, 8 and 6–7, respectively) exhibit buffering centered upon physiologic pH. As pH falls below 7.4, as in respiring tissue, amine protonation increases, displacing RSR13 from the inner shell, resulting in RSR13 ~ Hb binding in the EM cavity, raising P_{50} and facilitating O_2 release, particularly in tissues with aerobic insufficiency. As circulatory transit is completed in the lung (or, as hypoxia abates) and pH rises, RSR13 re-associates with increasingly available ionized precursor amines, thereby: (1) facilitating O_2 loading in the lung and (2) linking EM P_{50} to tissue O_2 need (Fig. 24.3). This physiologically responsive RSR13 shuttle-reservoir differentiates EM from all previously explored HBOC designs [100, 101].

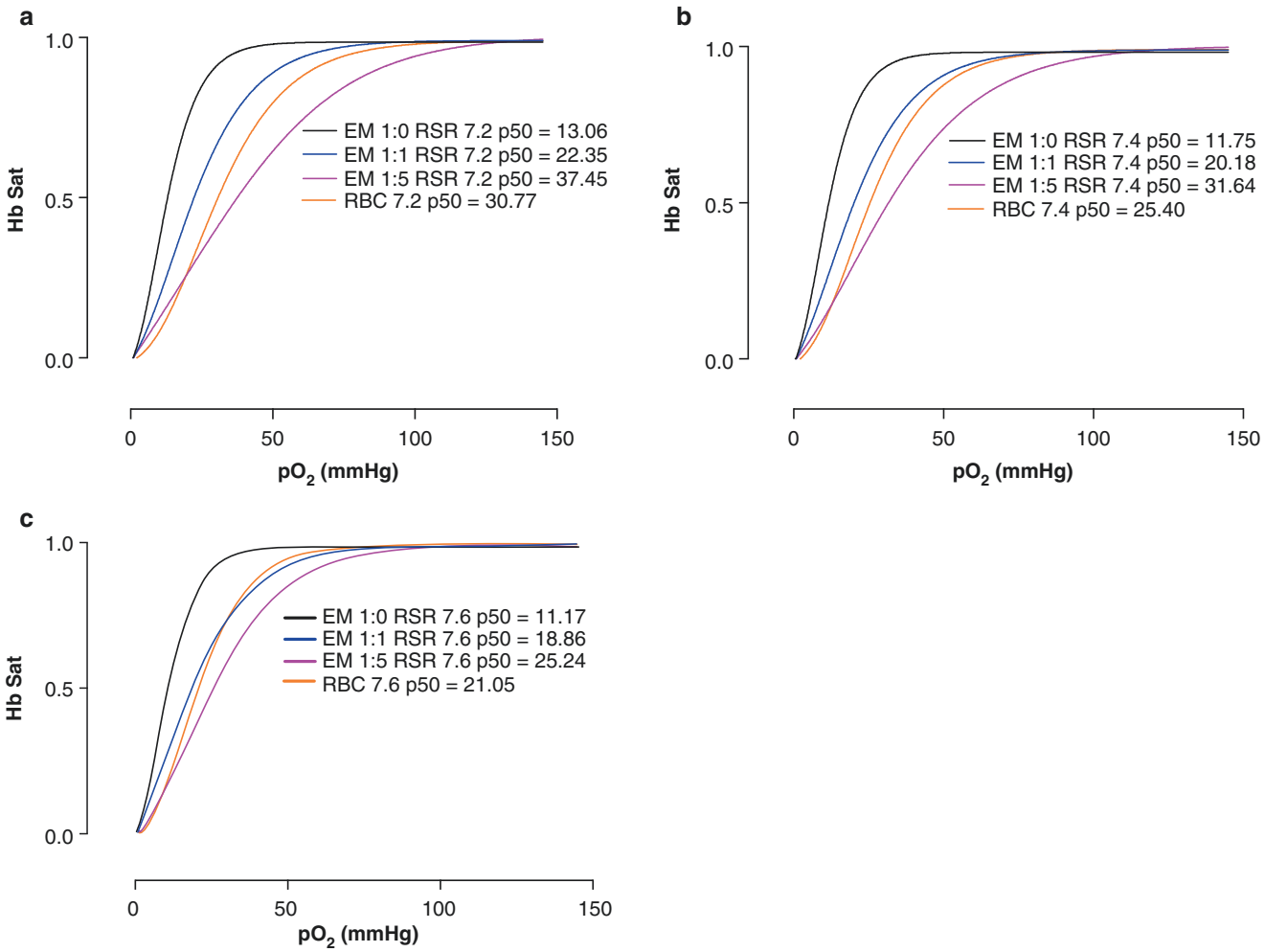


Fig. 24.3 O_2 Dissociation Curve (ODC) We measured Hb ~ O_2 equilibrium curves, for RBCs and EM (in Krebs, n = 5), (HEMOX analyzer). Relative to that for free Hb (black reference line) we observed near concordance between RBCs and EM for P_{50} at pH (**a**) 7.2, (**b**) 7.4 and (**c**) 7.6, illustrating physiologic pH responsive variation in O_2 affinity for EM. Moreover, by increasing the molar ratio of RSR13:Hb to 5:1 (lavender line), the ODC is right shifted relative to that for RBCs (tan line) resulting in more efficient release of O_2 in response to reduced pO_2. Importantly, the dynamic range for pH based variation in P_{50} was ~140% greater for RSR13:Hb @ 5:1 (Δ 10.85 Torr), than for RBCs (Δ 7.64 Torr), indicating: (1) that on a per gm Hb basis, ~ 140% more O_2 will flux via EM-based transit across any given O_2 gradient. Additionally, since EM is right-shifted relative to RBCs, EM may serve as an intravascular O_2 'shuttle' – facilitating O_2 flux from endogenous RBCs to respiring tissues, effectively using circulating RBCs as an in situ 'lung' in the microcirculation

Initial Biocompatibility (Complement)

In vitro analysis of EM-dependent activation of the human complement (C) system was tested. Total complement activity was measured using the CH50/hemolysis assay [102]. EM was incubated in human plasma and the degree of C activation is reflected by the hemolytic activity arising from generation of C3a and C5a fragments in the plasma supernatant. Attribution of hemolysis to C activation was confirmed by assay for C3a and C5a fragments by western blot and ELISA. These experiments were conducted across a range of EM:human serum ratios (1:8–1:64). The CH50 values were found to be: 2 ± 1; 5 ± 1; 2 ± 1; 8 ± 1; 9 ± 1 and 2 ± 1 for amines, non-cross-linked EM, cross-linked EM (at 60% colloidal suspension), fatty acid control, and for positive and negative controls, respectively. Importantly, EM lacks surface antigens and thus is a universal product that eliminates the need for donor/recipient compatibility testing.

Rheology

These properties of an O_2 carrier are critically important since the volume administered may alter blood viscosity and

hydrodynamics [103, 104]. EM influence upon plasma rheology was studied after suspension in NZW rabbit plasma, revealing only a minor reduction in suspension viscosity related to the dilution of the plasma by the aqueous media of the nanoparticles. The absence of viscosity increase by EM suggests that the nanoparticles do not aggregate in the presence of plasma proteins and will exert minimal influence on the rheological parameters of plasma.

Exploratory Pharmacokinetic (PK) Profiling in Rats and Rabbits

Our lead prototype, EMV2, was first evaluated in rats using two compartment nonlinear modeling (elimination $t_{1/2}$ of 26.2 ± 3.6 min). Of note, nanoparticle PK and Bio-D exhibit significant species-dependent variation; PK is accelerated significantly in rats (via biliary excretion). The EM circulatory $t_{1/2}$ in rats likely translates to a human $t_{1/2}$ of ~3 h based on allometric scaling. To increase the duration of exposure to EM, the lead prototype has been modified by substituting the amphiphilic polymer lipid with an oxidized form (1-lyso-2-acyl-sn-glycero-3-palimitoyl) and using probe sonication-facilitated self-assembly (4 °C), thereby increasing EM diameter (~25%↑, 220 nm) and shell stability. This size modification is known to strongly influence nanoparticle clearance [105–115], extended EM circulation in rats by ~135%, increasing the projected human $t_{1/2}$ to ~7 h. PK analysis in rabbits, a species with similar biliary clearance as humans, does not exhibit significant 'first-pass' NP excretion in bile [106]. The pharmacokinetic profile of EMV2 was determined in NZW rabbits (n = 9) using IRDye800 labeled EM (10% blood volume replacement) and PK metrics were determined with both noncompartmental (NC) and two-phase nonlinear (NL) modeling [116]. PK analysis demonstrated the following NC metrics (based upon EM particle #): Dose: $1.6 \times 10^{11} \pm 1.5 \times 10^{10}$; C_{MAX} $1.3 \times 10^{9} \pm 1.6 \times 10^{8}$; $AUC_{(0\text{-tlast})}$ $3.9 \times 10^{9} \pm 5.5 \times 10^{8}$; $AUC_{(0\text{-}\infty)}$ $4.45 \times 10^{9} \pm 7.4 \times 10^{8}$; $CL_{(total)}$ 36.2 ± 11.5 (mL/h); $t_{1/2}$ 2.1 ± 0.19, V_c 123.0 ± 16.9. *In silico* simulation based upon these NCA metrics identified the following dose interval requirements to maintain plasma EM [Hb] > 1 g/dL: 5, 7 and 8 h for C_{MAX} of 1, 2 or 3×10^{11} (EM particles/mL), respectively; these dose intervals are extended by a factor of 1 (reference), 2 and 3, for EM payload [Hb] of 1, 1.5, or 2 mM, respectively.

O_2 Delivery (*in vivo*)

EM has been evaluated in three acute models: hemorrhagic shock/resuscitation in rats; normotensive hemodilution in mice; and hemorrhagic shock in rabbits (Figs. 24.4 and 24.5). Results are encouraging and demonstrate robust efficacy compared to hydroxyethyl starch (HES) in rats and mice and albumin in resuscitated rabbits. In all studies, autologous shed blood was equivalent in efficacy to that of EM. In addition to conventional performance measures, a highly novel murine model, bioluminescence in HIF-1α (ODD)-luciferase mice [117–120], demonstrated integrated O_2 delivery/consumption balance and revealed constraint in O_2 delivery, regardless of step in this process (lung, heart, blood flow, HBOC or RBC loading/unloading). Demonstrating HIF-1α stabilization is the gold-standard measure of inadequate O_2 delivery to tissue; as such, we exploit this model to demonstrate EM ability to maintain tissue O_2 delivery during near-complete (70% volume) blood replacement. Exchange transfusion was selected (rather than hemorrhage/resuscitation) to avoid hypotension as a confounding cause for HIF-bioluminescence.

Envisioned Use

Pre-hospital care for civilian trauma remains a challenge, even in developed countries. 47 million Americans live more than 1 hour from a trauma center and most ambulances do not carry blood. Notably, while ~20% of the US population is rural, this group experiences 60% of trauma deaths. Trauma victims in hemorrhagic shock who do not receive blood until hospital arrival suffer mortality of 17–54% and mortality increases with time and distance to definitive care. The National Academy of Sciences has estimated that 30,000 US civilian trauma deaths/year occur are preventable, and that approximately 20,000 deaths that are preventable occur due to hemorrhage in the prehospital phase of resuscitation. Additionally, in an urban civilian disaster, delays in blood product readiness arise despite medical center proximity, further increasing mortality risk from hemorrhagic shock. As for military PFC settings, a field-deployable O_2 carrier that is compatible with lyophilized plasma and platelets will enable composition of a balanced resuscitation fluid that treats both shock and coagulopathy - and push advanced transfusion therapy for life-threatening civilian trauma to the field setting. A successful blood substitute, with easy in-field portability and administration may fundamentally change Resuscitation and Transfusion Medicine. Rather than replace/compete with human RBC transfusion, initial EM utilization is anticipated to be in pre-hospital, military and other austere environments, where conventional transfusion is not possible. In addition, EM properties (lack of vasoconstriction and immune reactivity) offer advantage over RBCs in certain hospital settings: (1) priming bypass circuits for cardiac surgery and (2) emergency use in bleeding, acutely decompensating patients, for whom

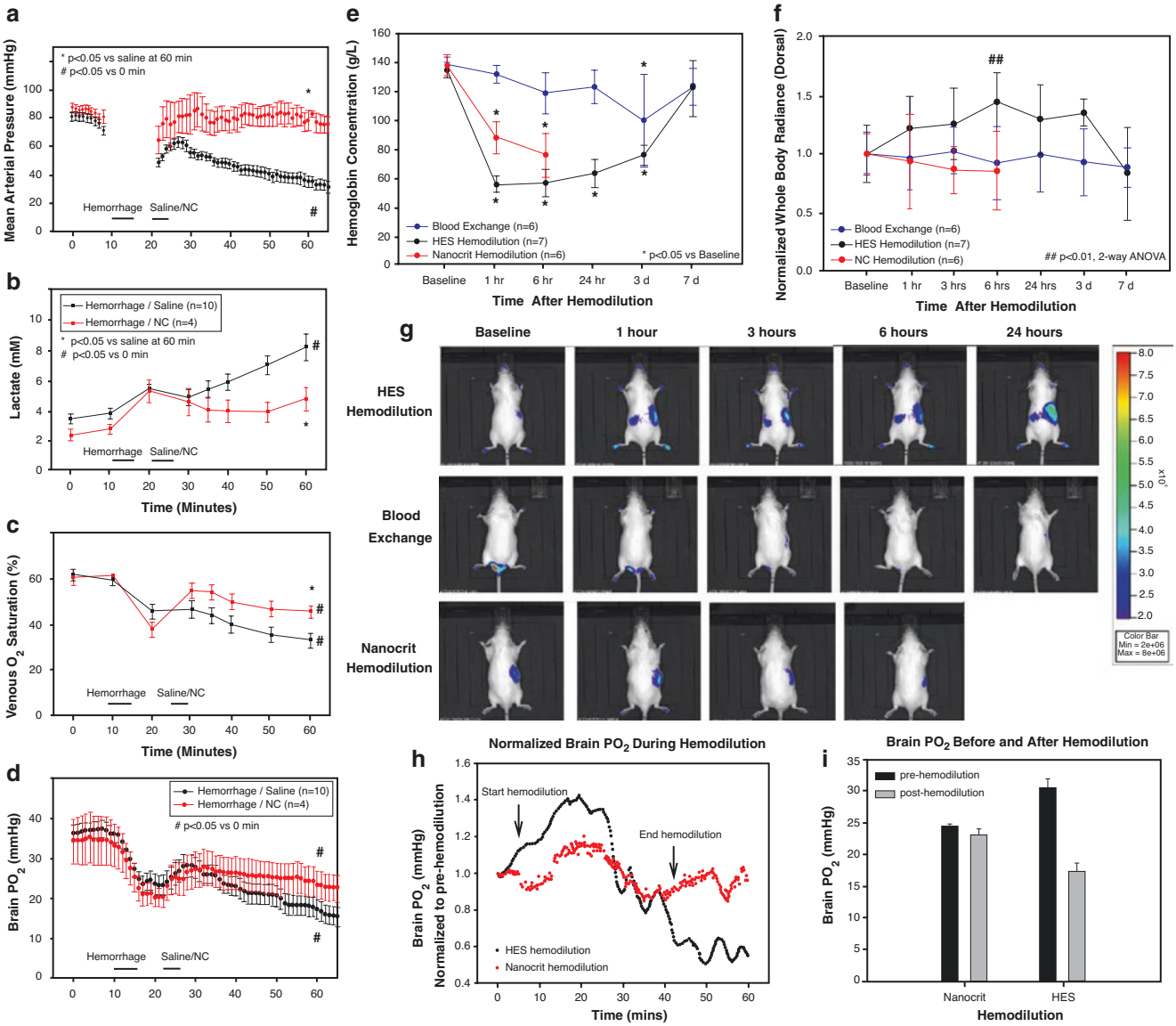


Fig. 24.4 EM *in vivo* O_2 delivery (A-D) rodent models: In fully instrumented SD Rats (400 g) 40% blood volume was removed; animals were then resuscitated with an equal volume of EM (n = 6) or normal saline (n = 6). EM was suspended at 40 wt/vol%, [Hb] = 4 mM. (**a**) EM infusion rapidly stabilized hemodynamics. (**b**) During the first hour, resolution of lactic acidosis (8.2 ± 2.1 v 3.2 ± 1.5 mM) and (**c**) elevated AV O_2 difference (67 ± 23 v 24 ± 11% Torr for EM and NS, respectively) were observed, as well as (**d**) improved brain pO_2 [$p < 0.05$, RMANOVA]. (**e–g**) Hemodilution model: Un-instrumented, HIF-1α (ODD) luciferase mice underwent hemodilution (70% v/v) with pentastarch, fresh blood (autotransfusion controls), or EM (n = 6); (**e**) Hb target nadir was reached (5 mg/dL). To detect whole body luciferase expression, D-luciferin (50 mg/kg, IP) was injected and serial images were obtained (IVIS, Living Image). (**f–g**) HIF-luc radiance was higher in the HES group than in blood exchange and EM groups, which did not differ ($p < 0.01$, RMANOVA). (**h**, **i**) Finally, during murine hemodilution (70% v/v), O_2 delivery by direct brain pO_2 measurement was demonstrated. In models of major bleeding/anemia, EM reconstitutes normal hemodynamics and O_2 delivery, observed at the system, tissue, and cellular level. NB: ErythroMer was formerly termed NanoCrit

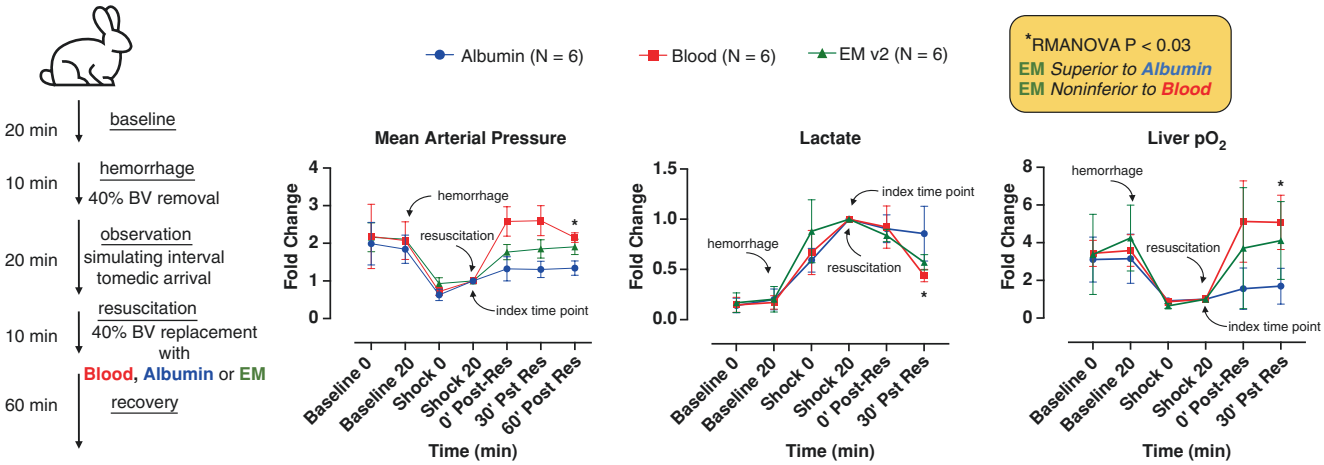


Fig. 24.5 Rabbit Model of Hemorrhagic Shock; resuscitation with whole blood, EM, or 5% Albumin. Hemorrhagic shock was induced by removal of 40% or 20% BV by wt, animals were allowed to stabilize for 20 min, then resuscitated by infusion of removed volume, with: shed blood (black); 5% human albumin (blue); EM 50 × 10^9 particles/mL, final (red); or EM 60 × 10^9 particles/mL, final (burgundy). Both EM preparations contained Hb:RSR13 payload at a ratio of 1:5. The figure captures (**right**) liver tissue pO_2, (**middle**) Lactate (mmol/L), and (**left**) mean arterial pressure (MAP), which demonstrate: (1) non-inferiority of EM-based resuscitation to re-infused shed blood (all parameters*) and (2) superiority of EM- to Albumin-based resuscitation

Table 24.3 Opportunities for ErythroMer use

Application		Location / Setting
General transfusion	**When stored RBCs are undesirable** Low-volume resuscitation Limit alloimmunization Limit transfusion immunomodulation Limit risk of infectious transmission	Pre-hospital, ED, OR, ICU, military environments
Hemorrhagic shock	**When stored RBCs are unavailable**	Pre-hospital, civilian disasters, military environments, undeveloped countries, complex cross-matches, use in austere or remote locations
Perioperative Maintenance of O_2 delivery	**When stored RBCs can be avoided** Brief periods of controlled blood loss	OR, ICU
Improve regional O_2 delivery	**Beyond conventional efficacy of stored RBCs** Target O_2 affinity to context Link pharmaceuticals to particle shell *Ex vivo* organ perfusion Nanoparticle perfusion through vascular obstruction (e.g. MI, stroke, PE, etc.)	Pre-hospital, ED, OR, ICU

cross-matching would delay resuscitation. We also anticipate interest in stockpiling EM in large-scale civilian emergency depots and developing world blood banks. See Table 24.3 for a more complete listing of envisioned opportunities of use. As noted above, an easily reconstituted blood substitute, amenable to extended ambient storage would have significant impact upon pre-hospital care for trauma victims in hemorrhagic shock, as well as enable creation of easily deployable 'strategic reserves' for blood bank support of disaster management. In addition, maintenance of a robust blood supply presents challenges in the developing world; specifically, creation and maintenance of processing and storage facilities for a decentralized blood bank system is not fiscally feasible. Moreover, in many countries, a pathogen-free donor pool is difficult to identify and maintain, and civil unrest leads to unpredictable surges in blood product requirements. For these reasons, a pathogen-free, lyophilized blood substitute would fulfill an unmet need in these areas as well.

Summary/Conclusion

There is a commonly appreciated, critical unmet need for a safe, effective, and practical O_2 transport agent to serve as an alternative to human RBCs. A successful artificial O_2 carrier must demonstrate context-responsive O_2 binding, without

NO sequestration. We have addressed this challenge by designing the only HBOC with highly novel, dynamic, context-responsive properties that imitate normal RBC physiology and optimize efficient O_2 transport during physiologic stress. As an Hb-encapsulating, toroidal-shaped nanoparticle formulated by self-assembly of amphiphilic polymer, EM will constitute a new class of formally engineered bio-synthetic hybrid 'artificial cells' (e.g., 'cell-mers'). The RBC-emulating physiologic performance of EM is an emergent property of biochemically encoded 'wetware'; this (integrated shell and payload) design strategy has the potential to disrupt and fundamentally alter our approach to numerous complex therapeutic challenges.

Key Points

- ErythroMer a first-in-class, bio-synthetic, toroidal nanocyte, red blood cell substitute
- The EM shell 'wetware' modulates O_2 affinity to context during circulation
- EM exhibits limited NO trapping and vasoactivity
- EM can be lyophilized enabling portability, facile reconstitution, and extended shelf life
- PK modelling indicates that in prolonged field care scenarios, a dosing interval of ~8–10 hours may maintain adequate tissue oxygen delivery
- In models of major bleeding/anemia, EM reconstitutes normal hemodynamics and O_2 delivery, observed at the system, tissue, and cellular level

References

1. Eichelbronner O, et al. Effects of FIO2 on hemodynamic responses and O2 transport during RSR13-induced reduction in P50. Am J Physiol. 1999;277:H290–8.
2. Grocott HP, et al. Effects of a synthetic allosteric modifier of hemoglobin oxygen affinity on outcome from global cerebral ischemia in the rat ï editorial comment. Stroke. 1998;29:1650–5.
3. Kilgore KS, et al. RSR13, a synthetic allosteric modifier of hemoglobin, improves myocardial recovery following hypothermic cardiopulmonary bypass. Circulation. 1999;100:35111–356.
4. Miyake M, et al. The effect of RSR13, a synthetic allosteric modifier of hemoglobin, on brain tissue pO2 (measured by EPR oximetry) following severe hemorrhagic shock in rats. Adv Exp Med Biol. 2003;530:319–29.
5. Steffen RP, Liard JF, Gerber MJ, Hoffman SJ. Allosteric modification of hemoglobin by RSR13 as a therapeutic strategy. Adv Exp Med Biol. 2003;530:249–59.
6. Weiss RG, et al. Preservation of canine myocardial high-energy phosphates during low-flow ischemia with modification of hemoglobinñoxygen affinity. J Clin Investig. 1999;103:739–46.
7. Chang TM. Hemoglobin-based red blood cell substitutes. Artif Organs. 2004;28:789–94. https://doi.org/10.1111/j.1525-1594.2004.07394.x.
8. Chen JY, Scerbo M, Kramer G. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. Clinics (Sao Paulo). 2009;64:803–13. https://doi.org/10.1590/S1807-59322009000800016.
9. Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E. Review of hemoglobin-vesicles as artificial oxygen carriers. Artif Organs. 2009;33:139–45. https://doi.org/10.1111/j.1525-1594.2008.00698.x.
10. Irwin DC, et al. Polymerized bovine hemoglobin decreases oxygen delivery during normoxia and acute hypoxia in the rat. Am J Physiol Heart Circ Physiol. 2008;295:H1090–9. https://doi.org/10.1152/ajpheart.00303.2008.
11. Cohn CS, Cushing MM. Oxygen therapeutics: perfluorocarbons and blood substitute safety. Crit Care Clin. 2009;25:399–414. https://doi.org/10.1016/j.ccc.2008.12.007.
12. Ross JM, Fairchild HM, Weldy J, Guyton AC. Autoregulation of blood flow by oxygen lack. Am J Physiol. 1962;202:21–4.
13. Singel DJ, Stamler JS. Blood traffic control. Nature. 2004;430:297.
14. Doctor A, Stamler JS. Nitric oxide transport in blood: a third gas in the respiratory cycle. Compr Physiol. 2011;1:611–38.
15. Hsia CCW. Respiratory function of hemoglobin. N Engl J Med. 1998;338:239–47.
16. Hsia CC, Coordinated W. Adaptation of oxygen transport in cardiopulmonary disease. Circulation. 2001;104:963–9.
17. Bunn HF, Briehl RW. The interaction of 2,3-diphosphoglycerate with various human hemoglobins. J Clin Invest. 1970;49:1088–95. https://doi.org/10.1172/JCI106324.
18. Dull RO, et al. Quantitative assessment of hemoglobin-induced endothelial barrier dysfunction. J Appl Physiol. 2004;97:1930–7. https://doi.org/10.1152/japplphysiol.00102.2004.
19. Faivre-Fiorina B, et al. Presence of hemoglobin inside aortic endothelial cells after cell-free hemoglobin administration in guinea pigs. Am J Phys. 1999;276:H766–70.
20. Smani Y, Faivre B, Audonnet-Blaise S, Labrude P, Vigneron C. Hemoglobin-based oxygen carrier distribution inside vascular wall and arterial pressure evolution: is there a relationship? Eur Surg Res. 2005;37:1–8. https://doi.org/10.1159/000083141.
21. Nakai K, et al. Permeability characteristics of hemoglobin derivatives across cultured endothelial cell monolayers. J Lab Clin Med. 1998;132:313–9.
22. Kavdia M, Tsoukias NM, Popel AS. Model of nitric oxide diffusion in an arteriole: impact of hemoglobin-based blood substitutes. Am J Physiol Heart Circ Physiol. 2002;282:H2245–53. https://doi.org/10.1152/ajpheart.00972.2001.
23. Hai CM. Systems biology of HBOC-induced vasoconstriction. Curr Drug Discov Technol. 2012;9:204–11.
24. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA. 2008;299:2304–12. https://doi.org/10.1001/jama.299.19.jrv80007.
25. Estep TN. Issues in the development of hemoglobin based oxygen carriers. Semin Hematol. 2019;56:257–61. https://doi.org/10.1053/j.seminhematol.2019.11.006.
26. Estep TN. Haemoglobin-based oxygen carriers and myocardial infarction. Artif Cells Nanomed Biotechnol. 2019;47:593–601. https://doi.org/10.1080/21691401.2019.1573181.
27. Buehler PW, et al. Structural and functional characterization of glutaraldehyde-polymerized bovine hemoglobin and its isolated fractions. Anal Chem. 2005;77:3466–78. https://doi.org/10.1021/ac050064y.
28. Alayash AI. Hemoglobin-based blood substitutes and the hazards of blood radicals. Free Radic Res. 2000;33:341–8.
29. Alayash AI. Oxygen therapeutics: can we tame haemoglobin? Nat Rev Drug Discov. 2004;3:152–9.
30. Buehler PW, Alayash AI. All hemoglobin based oxygen carriers are not created equally. Biochim Biophys Acta. 2008;1784:1378–81.

31. Meng F, et al. Comprehensive biochemical and biophysical characterization of hemoglobin-based oxygen carrier therapeutics: all HBOCs are not created equally. Bioconjug Chem. 2018;29:1560–75. https://doi.org/10.1021/acs.bioconjchem.8b00093.
32. Alayash AI. Mechanisms of toxicity and modulation of hemoglobin-based oxygen carriers. Shock. 2019;52:41–9. https://doi.org/10.1097/SHK.0000000000001044.
33. Pittman RN. Oxygen transport and exchange in the microcirculation. Microcirculation. 2005;12:59–70. https://doi.org/10.1080/10739680590895064.
34. Tsai AG, Johnson PC, Intaglietta M. Oxygen gradients in the microcirculation. Physiol Rev. 2003;83:933–63. https://doi.org/10.1152/physrev.00034.2002.
35. Raat NJ, Ince C. Oxygenating the microcirculation: the perspective from blood transfusion and blood storage. Vox Sang. 2007;93:12–8. https://doi.org/10.1111/j.1423-0410.2007.00909.x.
36. Jahr JS, Akha AS, Holtby RJ. Crosslinked, polymerized, and PEG-conjugated hemoglobin-based oxygen carriers: clinical safety and efficacy of recent and current products. Curr Drug Discov Technol. 2012;9:158–65.
37. Jahr JS, Walker V, Manoochehri K. Blood substitutes as pharmacotherapies in clinical practice. Curr Opin Anaesthesiol. 2007;20:325–30. https://doi.org/10.1097/ACO.0b013e328172225a.
38. Mozzarelli A, Ronda L, Faggiano S, Bettati S, Bruno S. Haemoglobin-based oxygen carriers: research and reality towards an alternative to blood transfusions. Blood Transf. 2010;8 Suppl 3, s59–68. https://doi.org/10.2450/2010.010S.
39. Piras AM, et al. Polymeric nanoparticles for hemoglobin-based oxygen carriers. Biochim Biophys Acta. 2008;1784:1454–61. https://doi.org/10.1016/j.bbapap.2008.03.013.
40. Sakai H. Present situation of the development of cellular-type hemoglobin-based oxygen carrier (hemoglobin-vesicles). Curr Drug Discov Technol. 2012;9:188–93.
41. Silverman TA, Weiskopf RB. Hemoglobin-based oxygen carriers: current status and future directions. Transfusion. 2009;49:2495–515. https://doi.org/10.1111/j.1537-2995.2009.02356.x.
42. Napolitano LM. Hemoglobin-based oxygen carriers: first, second or third generation? Human or bovine? Where are we now? Crit Care Clin. 2009;25:279–301. https://doi.org/10.1016/j.ccc.2009.01.003.
43. Pan DPJRS, Misra S, Vulugundam G, Gazdzinski L, Tsui A, Mistry N, Said A, Spinella P, Hare G, Lanza G, Doctor A. Erythromer (EM), a nanoscale bio-synthetic artificial red cell: proof of concept and in vivo efficacy results. Blood. 2016;128:A0127.
44. Safo MK, Ahmed MH, Ghatge MS, Boyiri T. Hemoglobin-ligand binding: understanding Hb function and allostery on atomic level. Biochim Biophys Acta. 2011;1814:797–809. https://doi.org/10.1016/j.bbapap.2011.02.013.
45. Richardson RS, Tagore K, Haseler LJ, Jordan M, Wagner PD. Increased VO2 max with right-shifted Hb-O2 dissociation curve at a constant O2 delivery in dog muscle in situ. J Appl Physiol 1985. 1998;84:995–1002. https://doi.org/10.1152/jappl.1998.84.3.995.
46. Najjar SS, et al. Effects of a pharmacologically-induced shift of hemoglobin-oxygen dissociation on myocardial energetics during ischemia in patients with coronary artery disease. J Cardiovasc Magn Reson. 2005;7:657–66.
47. Wahr JA, Gerber M, Venitz J, Baliga N. Allosteric modification of oxygen delivery by hemoglobin. Anesth Analg. 2001;92:615–20. https://doi.org/10.1097/00000539-200103000-00011.
48. Khandelwal SR, et al. Enhanced oxygenation in vivo by allosteric inhibitors of hemoglobin saturation. Am J Phys. 1993;265:H1450–3. https://doi.org/10.1152/ajpheart.1993.265.4.H1450.
49. Watanabe T, et al. Reduction in hemoglobin-oxygen affinity results in the improvement of exercise capacity in mice with chronic heart failure. J Am Coll Cardiol. 2008;52:779–86. https://doi.org/10.1016/j.jacc.2008.06.003.
50. Isbell TS, et al. Assessing NO-dependent vasodilatation using vessel bioassays at defined oxygen tenstions. Methods Enzymol. 2005;396:553–68.
51. Marden MC, Griffon N, Poyart C. Oxygen delivery and autoxidation of hemoglobin. Transfus Clin Biol. 1995;2:473–80.
52. Rogers SC, et al. Hypoxia limits antioxidant capacity in red blood cells by altering glycolytic pathway dominance. FASEB J. 2009;23:3159–70. https://doi.org/10.1096/fj.09-130666.
53. Siems WG, Sommerburg O, Grune T. Erythrocyte free radical and energy metabolism. Clin Nephrol. 2000;53:S9–17.
54. Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E. Hemoglobin-vesicle, a cellular artificial oxygen carrier that fulfils the physiological roles of the red blood cell structure. Adv Exp Med Biol. 2010;662:433–8. https://doi.org/10.1007/978-1-4419-1241-1_62.
55. Sakai H, Sou K, Tsuchida E. Hemoglobin-vesicles as an artificial oxygen carrier. Methods Enzymol. 2009;465:363–84. https://doi.org/10.1016/S0076-6879(09)65019-9.
56. Sakai H, Tsuchida E. Performances of PEG-modified hemoglobin-vesicles as artificial oxygen carriers in microcirculation. Clin Hemorheol Microcirc. 2006;34:335–40.
57. Sakai H, Yuasa M, Onuma H, Takeoka S, Tsuchida E. Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. Bioconjug Chem. 2000;11:56–64.
58. Takeoka S, et al. Construction of artificial methemoglobin reduction systems in Hb vesicles. Artif Cells Blood Substit Immobil Biotechnol. 1997;25:31–41.
59. Takeoka S, et al. Methemoglobin formation in hemoglobin vesicles and reduction by encapsulated thiols. Bioconjug Chem. 1997;8:539–44. https://doi.org/10.1021/bc970091y.
60. Atoji T, Aihara M, Sakai H, Tsuchida E, Takeoka S. Hemoglobin vesicles containing methemoglobin and L-tyrosine to suppress methemoglobin formation in vitro and in vivo. Bioconjug Chem. 2006;17:1241–5. https://doi.org/10.1021/bc050349h.
61. Teramura Y, Kanazawa H, Sakai H, Takeoka S, Tsuchida E. Prolonged oxygen-carrying ability of hemoglobin vesicles by coencapsulation of catalase in vivo. Bioconjug Chem. 2003;14:1171–6. https://doi.org/10.1021/bc0340619.
62. Zhang X, et al. Reduction and suppression of methemoglobin loaded in the polymeric nanoparticles intended for blood substitutes. J Biomed Mater Res B Appl Biomater. 2008;87:354–63. https://doi.org/10.1002/jbm.b.31110.
63. Blank O, Davioud-Charvet E, Elhabiri M. Interactions of the antimalarial drug methylene blue with methemoglobin and heme targets in Plasmodium falciparum: a physico-biochemical study. Antioxid Redox Signal. 2012;17:544–54. https://doi.org/10.1089/ars.2011.4239.
64. Sass MD, Caruso CJ, Axelrod DR. Accumulation of methylene blue by metabolizing erythrocytes. J Lab Clin Med. 1967;69:447–55.
65. Sass MD, Caruso CJ, Axelrod DR. Mechanism of the TPNH-linked reduction of methemoglobin by methylene blue. Clin Chim Acta. 1969;24:77–85.
66. May JM, Qu ZC, Cobb CE. Reduction and uptake of methylene blue by human erythrocytes. Am J Physiol Cell Physiol. 2004;286:C1390–8. https://doi.org/10.1152/ajpcell.00512.2003.
67. Auker CR, McCarron RM. US Navy experience with research on, and development of, hemoglobin-based oxygen carriers. J Trauma. 2011;70:S40–1. https://doi.org/10.1097/TA.0b013e31821a5a1e.
68. de Figueiredo LF. Vasoactive properties of synthetic blood substitutes. Medicina. 1998;58:403–10.
69. Yu B, et al. Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobin-based oxygen car-

rier. Anesthesiology. 2010;112:586–94. https://doi.org/10.1097/ALN.0b013e3181cd7838.
70. Moschandreou TE, Ellis CG, Goldman D. Influence of tissue metabolism and capillary oxygen supply on arteriolar oxygen transport: a computational model. Math Biosci. 2011;232:1–10. https://doi.org/10.1016/j.mbs.2011.03.010.
71. Chen X, Buerk DG, Barbee KA, Jaron D. A model of NO/O2 transport in capillary-perfused tissue containing an arteriole and venule pair. Ann Biomed Eng. 2007;35:517–29. https://doi.org/10.1007/s10439-006-9236-z.
72. Cabrales P, Han G, Nacharaju P, Friedman AJ, Friedman JM. Reversal of hemoglobin-induced vasoconstriction with sustained release of nitric oxide. Am J Physiol Heart Circ Physiol. 2011;300:H49–56. https://doi.org/10.1152/ajpheart.00665.2010.
73. Yu B, et al. Inhaled nitric oxide enables artificial blood transfusion without hypertension. Circulation. 2008;117:1982–90. https://doi.org/10.1161/CIRCULATIONAHA.107.729137.
74. Rodriguez C, et al. Sodium nitrite therapy attenuates the hypertensive effects of HBOC-201 via nitrite reduction. Biochem J. 2009;422:423–32. https://doi.org/10.1042/BJ20090735.
75. Irwin D, et al. Mixed S-nitrosylated polymerized bovine hemoglobin species moderate hemodynamic effects in acutely hypoxic rats. Am J Respir Cell Mol Biol. 2010;42:200–9. https://doi.org/10.1165/rcmb.2008-0364OC.
76. Nakai K, et al. Preparation and characterization of SNO-PEG-hemoglobin as a candidate for oxygen transporting material. Int J Artif Organs. 2001;24:322–8.
77. Asanuma H, et al. S-nitrosylated and pegylated hemoglobin, a newly developed artificial oxygen carrier, exerts cardioprotection against ischemic hearts. J Mol Cell Cardiol. 2007;42:924–30. https://doi.org/10.1016/j.yjmcc.2006.12.001.
78. Katz LM, et al. Nitroglycerin attenuates vasoconstriction of HBOC-201 during hemorrhagic shock resuscitation. Resuscitation. 2010;81:481–7. https://doi.org/10.1016/j.resuscitation.2009.12.015.
79. Ellis CG, Bateman RM, Sharpe MD, Sibbald WJ, Gill R. Effect of a maldistribution of microvascular blood flow on capillary O2 extraction in sepsis. Am J Physiol Heart Circ Physiol. 2002;282:H156–64.
80. Gonzalez-Alonso J, Mortensen SP, Dawson EA, Secher NH, Damsgaard R. Erythrocytes and the regulation of human skeletal muscle blood flow and oxygen delivery: role of erythrocyte count and oxygenation state of haemoglobin. J Physiol. 2006;572:295–305.
81. Tsoukias NM. Nitric oxide bioavailability in the microcirculation: insights from mathematical models. Microcirculation. 2008;15:813–34.
82. Tsoukias NM, Popel AS. Erythrocyte consumption of nitric oxide in presence and absence of plasma-based hemoglobin. Am J Physiol Heart Circ Physiol. 2002;282:H2265–77. https://doi.org/10.1152/ajpheart.01080.2001.
83. Doctor A, et al. Hemoglobin conformation couples erythrocyte S-nitrosothiol content to O2 gradients. Proc Natl Acad Sci. 2005;102:5709–14.
84. Gaston B, Singel D, Doctor A, Stamler JS. S-nitrosothiol signaling in respiratory biology. Am J Respir Crit Care Med. 2006;173:1186–93.
85. McMahon TJ, et al. Nitric oxide in the human respiratory cycle. Nat Med. 2002;8:711–7.
86. Deem S. Nitric oxide scavenging by hemoglobin regulates hypoxic pulmonary vasoconstriction. Free Radic Biol Med. 2004;36:698–706. https://doi.org/10.1016/j.freeradbiomed.2003.11.025.
87. McMahon TJ, Exton SA, Bonaventura J, Singel DJ, Solomon SJ. Functional coupling of oxygen binding and vasoactivity in S-nitrosohemoglobin. J Biol Chem. 2000;275:16738–45.
88. Liu L, et al. Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. Cell. 2004;116:617–28.
89. Diesen DL, Hess DT, Stamler JS. Hypoxic vasodilation by red blood cells: evidence for an S-nitrosothiol-based signal. Circ Res. 2008;103:545–53.
90. Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. Nature. 1996;380:221–6.
91. Stamler JS, et al. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. Science. 1997;276:2034–7.
92. Singel DJ, Stamler JS. Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin. Annu Rev Physiol. 2005;67:99–145.
93. Singel DJ, Stamler JS. Chemical physiology of blood flow regulation by red blood cells: role of nitric oxide and S-nitrosohemoglobin. Annu Rev Physiol. 2005;67:99–145.
94. Han TH, Liao JC. Erythrocyte nitric oxide transport reduced by a submembrane cytoskeletal barrier. Biochim Biophys Acta Gen Subj. 2005;1723:135–42.
95. Han TH, et al. Regulation of nitric oxide consumption by hypoxic red blood cells. Proc Natl Acad Sci. 2003;100:12504–9.
96. Huang KT, et al. Modulation of nitric oxide bioavailability by erythrocytes. Proc Natl Acad Sci. 2001;98:11771–6.
97. Vaughn MW, Huang KT, Kuo L, Liao JC. Erythrocytes possess an intrinsic barrier to nitric oxide consumption. J Biol Chem. 2000;275:2342–8.
98. Vaughn MW, Huang KT, Kuo L, Liao JC. Erythrocyte consumption of nitric oxide: competition experiment and model analysis. Nitric Oxide. 2001;5:18–31.
99. Vaughn MW, Kuo L, Liao JC. Effective diffusion distance of nitric oxide in the microcirculation. Am J Physiol. 1998;274:H1705–14.
100. Abraham DJ, et al. Allosteric modifiers of hemoglobin: 2-[4-[[(3,5-disubstituted anilino)carbonyl]methyl]phenoxy]-2-methylpropionic acid derivatives that lower the oxygen affinity of hemoglobin in red cell suspensions, in whole blood, and in vivo in rats. Biochemistry. 1992;31:9141–9. https://doi.org/10.1021/bi00153a005.
101. Abraham RZ, Kobzik L, Moody MR, Reid MB, Stamler JS. Cyclic GMP is a second messenger by which nitric oxide inhibits diaphragm contraction. Comp Biochem Physiol A Mol Integr Physiol. 1998;119:177–83.
102. Nascimento P Jr, de Paiva Filho O, de Carvalho LR, Braz JR. Early hemodynamic and renal effects of hemorrhagic shock resuscitation with lactated Ringer's solution, hydroxyethyl starch, and hypertonic saline with or without 6% dextran-70. J Surg Res. 2006;136:98–105. https://doi.org/10.1016/j.jss.2006.04.021.
103. Hare GM, et al. Hemodilutional anemia is associated with increased cerebral neuronal nitric oxide synthase gene expression. J Appl Physiol. 2003;94:2058–67. https://doi.org/10.1152/japplphysiol.00931.2002.
104. Mazer CD, et al. Increased cerebral and renal endothelial nitric oxide synthase gene expression after cardiopulmonary bypass in the rat. J Thorac Cardiovasc Surg. 2007;133:13–20. https://doi.org/10.1016/j.jtcvs.2006.06.047.
105. Brooks MB, Stokol T, Catalfamo JL. Comparative hemostasis: animal models and new hemostasis tests. Clin Lab Med. 2011;31:139–59. https://doi.org/10.1016/j.cll.2010.10.009.
106. Goldsby RA, Kindt TK, Osborne BA, Kuby J. Immunology. 5th ed. New York: W.H. Freeman and Company; 2003.
107. Boura C, et al. Volume expansion with modified hemoglobin solution, colloids, or crystalloid after hemorrhagic shock in rabbits: effects in skeletal muscle oxygen pressure and use versus arterial blood velocity and resistance. Shock. 2003;19:176–82.
108. Jiang S, et al. Therapeutic mild hypothermia improves early outcomes in rabbits subjected to traumatic uncontrolled hemorrhagic

shock. J Surg Res. 2013;179:145–52. https://doi.org/10.1016/j.jss.2012.09.024.
109. Terajima K, Tsueshita T, Sakamoto A, Ogawa R. Fluid resuscitation with hemoglobin vesicles in a rabbit model of acute hemorrhagic shock. Shock. 2006;25:184–9. https://doi.org/10.1097/01.shk.0000192118.68295.5d.
110. Zhang YM, Gao B, Wang JJ, Sun XD, Liu XW. Effect of hypotensive resuscitation with a novel combination of fluids in a rabbit model of uncontrolled hemorrhagic shock. PLoS One. 2013;8:e66916. https://doi.org/10.1371/journal.pone.0066916.
111. Lhuillier F, et al. Nitric oxide and liver microcirculation during autoregulation and haemorrhagic shock in rabbit model. Br J Anaesth. 2006;97:137–46. https://doi.org/10.1093/bja/ael097.
112. Briet F, et al. Cerebral cortical gene expression in acutely anemic rats: a microarray analysis. Can J Anaesth. 2009;56:921–34. https://doi.org/10.1007/s12630-009-9201-z.
113. Hare GM. Tolerance of anemia: understanding the adaptive physiological mechanisms which promote survival. Trans Apher Sci. 2014;50:10–2. https://doi.org/10.1016/j.transci.2013.12.005.
114. Hare GM, et al. Effect of oxygen affinity and molecular weight of HBOCs on cerebral oxygenation and blood pressure in rats. Can J Anaesth. 2006;53:1030–8. https://doi.org/10.1007/BF03022533.
115. Hare GM, et al. Increased cerebral tissue oxygen tension after extensive hemodilution with a hemoglobin-based oxygen carrier. Anesth Analg. 2004;99:528–35. https://doi.org/10.1213/01.ANE.0000136769.65960.D1.
116. Dougherty SWQ, Rogers S, Moitra P, Yildiz T, Brummet M, Shea S, Thomas K, Buehler P, Spinella P, Pan D, Doctor A. Military Health System Research Symposium (MHSRS). US Department of Defense: Orlando; 2021.
117. McIntyre LA, et al. Effect of a liberal versus restrictive transfusion strategy on mortality in patients with moderate to severe head injury. Neurocrit Care. 2006;5:4–9.
118. McLaren AT, et al. Increased expression of HIF-1alpha, nNOS, and VEGF in the cerebral cortex of anemic rats. Am J Physiol Regul Integr Comp Physiol. 2007;292:R403–14. https://doi.org/10.1152/ajpregu.00403.2006.
119. Ragoonanan TE, et al. Metoprolol reduces cerebral tissue oxygen tension after acute hemodilution in rats. Anesthesiology. 2009;111:988–1000. https://doi.org/10.1097/ALN.0b013e3181b87f0e.
120. Rigamonti A, et al. Storage of strain-specific rat blood limits cerebral tissue oxygen delivery during acute fluid resuscitation. Br J Anaesth. 2008;100:357–64. https://doi.org/10.1093/bja/aem401.

OxyVita: History, Studies, and Future

25

Hanna Wollocko, Jacek Wollocko, Jonathan S. Jahr, and Kenneth Steier

Background

OxyVita is one of the polymeric hemoglobin-based oxygen carriers (HBOC), it was invented by the team of Professor Enrico Bucci at the University of Maryland in 1999. OxyVita does not have blood type, just like other HBOCs. It is presently prepared from bovine hemoglobin; however, any mammalian hemoglobin could be used for this purpose. The raw materials are readily available and ubiquitously existing. The zero-link polymerization technology is a well-known technique in biochemistry and the pharmaceutical industry. However, prior to OxyVita, this technique has never been applied for hemoglobin polymerization. During the process of utilizing the zero-link polymerization technique, only activators are used to create a macropolymer of hemoglobin, rather than utilizing linking agents. Therefore, the Zero-link polymerization does not leave any residual chemicals/polymerization agents/linking agents in the final molecule.

OxyVita is formulated to have an extended shelf life and it can be powderized. Its development and testing process have spanned over 20 years, with significant accomplishments and accolades in the field. The key to the success of the OxyVita product is a well-controlled design and its manufacturing process, allowing the product to be tailored to meet various needs in human and veterinary applications, such as a treatment in hemorrhage, traumatic brain injury, myocardial ischemia, sickle cell anemia, and many other clinical situations, as proposed further within this chapter.

The product has undergone a variety of investigations over the time of its life-span with a significant portion of studies being performed by third party researchers and not funded by the manufacturing company. The developmental evolution of the product has had significant influences of some prominent researchers. These previous research findings have laid the foundation to thoroughly assess the potential of the product and its potential future clinical applications.

Preparation of OxyVita

Detailed description of the process and zero-link polymerization technique can be found in literature [1, 2].

Briefly, the technique includes the following processes:

1. Raw material: bovine blood, but any mammalian blood can be used.
2. Preparation of raw material: centrifugation and lysis of red blood cells (RBC). This material is processed using several runs of centrifugation with 3000RPM - 4000RPM, to ensure the highest purity.
3. The Hb obtained is thereafter β-β cross-linked [bis(3,5dibromosalicyl-adipate)] to stabilize the hemoglobin tetramers.
4. Polymerization is then initiated using the carbodiimide, EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide], which is responsible for the activation of the side chain carboxylate groups on the hemoglobin surface. A complex is formed from the C- terminal on Glu and Asp globin side chain carboxylate groups. These activated species then react with the side chain of the lysyl residues of an adjacent hemoglobin tetrameric molecule to form a stable amide bond (covalent), referred to as a pseudo-peptide bond [3].

H. Wollocko (✉)
OXYVITA Inc., Middletown, NY, USA

Touro College of Osteopathic Medicine, Middletown, NY, USA
e-mail: hannaw@oxyvita.us

J. Wollocko
OXYVITA Inc., Middletown, NY, USA
e-mail: jacekw@oxyvita.us

J. S. Jahr
Department of Anesthesiology and Perioperative Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
e-mail: j.s.jahr@ucla.edu

K. Steier
Touro College of Osteopathic Medicine, Middletown, NY, USA
e-mail: kenneth.steier@touro.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_25

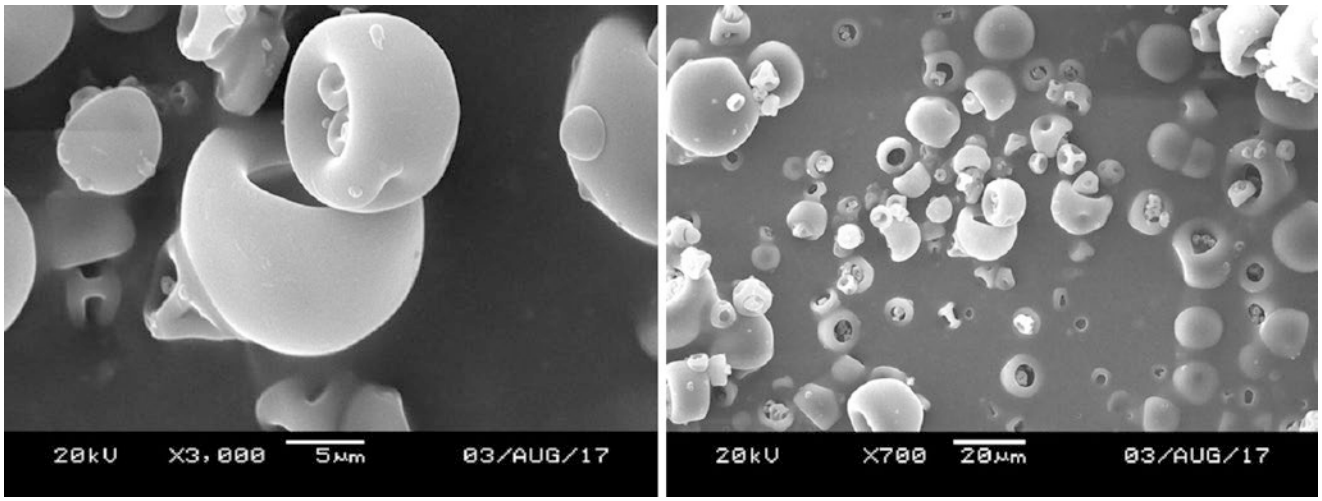


Fig. 25.1 Scanning Electron Microscopy (SEM) of OxyVita powder

Table 25.1 OxyVita Solution vs OxyVita Reconstituted Powder

OxyVita®C Solution results	Analysis	Reconstituted powder of OxyVita®C, results
6.35g%	g% of Hb Ferric (Fe^{2+})[a]	6.30g%
0.35g%	g% of Met Hb Ferrous (Fe^{3+})[a]	0.39g%
0.03g%	g% of Ferryl (Fe^{4+})[a]	0.04g%
100%	Size Exclusion Chromatography (SEC)[b] – % of POLYMER	100%
0.0	Size Exclusion Chromatography (SEC)[b] – % of Tetramer (64MDa)	0.0
0.0	Size Exclusion Chromatography (SEC)[b] – % of other sizes (below 64MDa)	0.0
312	Osmolarity[c] mOsm/kg	335
1.013	Density of solutions[d] g/mL	1.045
0.975	Spectral Radio[a] A_{576}/A_{540}	0.965
5.95 360	Oxygen Affinity[e] P_{50} Average Hydrodynamic Radius[f] Å	6.59 359
17.453	Average Molecular Weight[f] MDa	17.337
7.295	pH	7.353
N/A	Reconstitution Time sec.	<60

[a]UV/Vis HP Spectrophotometer, [b]LC with 600 mm × 15 mm column, [c]Osmometer –FLEX, [d]Oswald densitometer, [e]Hemox Analyzer (T-37°C, pH = 7.50), [f]Dyna-Pro 801 (Protein Solution)

5. N-hydroxysulfosuccinimide (sulfo-NHS) is introduced to the carbodiimide reaction, and results in the formation of an intermediate sulfo-NHS ester, which then reacts with the amino groups [4]. By altering the relative amounts of sulfo-NHS and EDC within the reaction mixture, the extent of the polymerization process can be manipulated, allowing for better control of the average molecular sizes during the manufacturing process.
6. The powder form of OxyVita Hb (Fig. 25.1) is produced by either lyophilization or spray-drying of the liquid form of OxyVita. After re-constitution, the powder has almost identical characteristics to the solution from which it is derived. The comparison of the characteristics of both liquid and powder forms of OxyVita is presented below (Table 25.1). The powder's shelf -life is 5 years under a wide range of climatic conditions.

The original preparation contained a higher amount of heterogeneous distribution of high molecular weight (MW) species with an average of 25 MDa [5, 6]. OXYVITA, Inc. acquired the license for commercial manufacturing from the University of Maryland, and, thereafter, refined the process, mostly to achieve higher homogeneity, as well as adjusting the average MW of polymer.

The Properties of OxyVita

Understanding the relationship between the structure and the function of oxygen carriers was pivotal in the development of this product. OxyVita belongs to the newer generation of hemoglobin-derived oxygen carriers. Thus, during the development of OxyVita, we have been able to benefit from the lessons learned from the development of earlier products. Specifically, we are more aware of, and paid more attention to, the potential side effects of extravasation, vasoconstriction and oxidative events, which were prevalent in the previous generations of similar products. OxyVita was therefore specifically designed with the aim of avoiding these side effects.

The controlled process of zero-link polymerization allows for specific, pre-defined characteristics, such as a specific MW and hydrodynamic radius of the product, a negative sur-

face charge, precise oxygen affinity (P_{50}), as well as viscosity similar to plasma and a relatively long retention time (Table 25.1).

The characteristics of OxyVita are integral to the product's functional attributes. The polymer is very stable, due to its intramolecular and intermolecular bonds; strong amide bonds connect tetrameric, previously cross-linked hemoglobin molecules into a polymer; on average, there are around 1000 tetramers per polymer. The specific crosslinking on β-β lysines 82 further stabilizes secondary and tertiary structures of the natural bovine hemoglobin, which results in increased conformational stability with a resistance to unfolding. Thanks to this, the heme-iron moieties get increased protection against molecular unfolding, which would otherwise result in heme exposure and iron loss, and consequently the loss of the oxygen carrier's effectiveness in its ability to carry oxygen. Previous studies on the structural integrity of OxyVita and several other natural hemoglobins, proved OxyVita's reduced tendency to unfold. This was done through an investigation of changes in the Soret region (350–450 nm), which is extremely sensitive to alterations within the heme [1, 7, 8].

The molecular size of OxyVita, consistently an average of 17MD, with an average hemodynamic radius of 360 Å and its negative charge on the polymer's surface, prevents extravasation by simply following the rules of physics, specifically, similar charges repel and size exclusion. The polymer is larger than any pores in the vasculature, therefore, it doesn't extravasate. As the polymer stays within the vasculature upon the injection, it doesn't scavenge NO from the external walls of blood vessels, in turn, causing no changes in mean arterial blood pressure (MAP). This has been shown through animal studies reporting the maintenance of the mean arterial pressure, in exchange transfusion and top-load studies, amongst others, while oxygenation of tissue was achieved [9, 10].

The P_{50} of the product is low, at 4–6 mmHg, compared to RBCs and several other products. This design's intent is facilitating oxygen release in microcirculation. Low P_{50} correlates to high oxygen affinity, meaning oxygen release occurs only with the presence of oxygen debt in the tissue, so, not in the macro-circulation.

Selected Studies

Years of in vitro and in-vivo studies with OxyVita brought significant insight into the mechanisms and potential physiologic and pharmacological features of this product. The performed studies have served as an assessment of the safety profile of the product for the potential future application for human and veterinary usage.

Summarizing the published studies conducted, work was performed on assessment of vascular response to infusion of polymer [9], cerebral ischemia and blood flow [11, 12], resuscitation [13], coagulation behavior [14] and redox behaviour [8], among other physiological effects of polymer action. We've learned that upon use of these large polymeric hemoglobin molecules, no hemoglobin was evident in the hilar lymph of rats and no increase in MAP was observed within both anesthetized and awake cats [9]. The study by Mito et al. [11] showed a reduction of cerebral infarct size by 39% in the mouse brain when treated with OxyVita. This beneficial effect seems to be dependent upon the concentration of this high-affinity hemoglobin polymer (P_{50} = 4 mm Hg), with 6% being the ideal concentration, while lower than 3% having no beneficial effect, and the molecular size of the hemoglobin polymers, with intermediate size being the best. It is likely that the imtermediate size could be better adjusted to the mouse's circulatory system [11]. In a DARPA small volume resuscitation study with a rat hemorrhagic shock model induced by bleeding 60% of total blood volume, an OxyVita-augmented hypertonic "cocktail" was proven to be a viable treatment for improved survival and MAP support [13]. Jahr et al. [14] investigated the impact of OxyVita on blood coagulation and found that minimal coagulopathic effects should be expected with the use of OxyVita at the anticipated effective dose of 10 g or 2-3 ml/kg. Bucci et al.[4]concluded that the extravasation and significant MAP increase were avoided when using the hemoglobin polymer with an average MW of 25 MDa and P_{50} of 18-30 mmHg. Their study showed that OxyVita delivered oxygen to tissue, providing either vasodilation or vasoconstriction according to oxygen needs *in vivo*. These studies also showed that cell free hemoglobins are potentially more efficient oxygen carrier than RBCs for delivering oxygen to tissue. Harrington et al. [7] conducted a redox behavior comparison study of OxyVita and natural acellular hemoglobin, *Lumbricus terrestris* Hb and *Arenicola marina* Hb, confirming that structural integrity in the reduced state (heme-Fe^{+2}) of OxyVita Hb is evident by its greater resistance to molecular unfolding than either of these natural hemoglobins; the reduction of met (heme-Fe^{+3}) OxyVita Hb to oxyHb occurs slowly in the presence of either ascorbic acid (70% reduction in 560 minutes) or β-NADH (40% reduction in 90 minutes). Wollocko et al. [8] studied the impact of oxidative stress by comparing OxyVita to myoglobin and natural bovine Hb, when exposed to unfolding agents. Little or no change in the Soret Maxima, small decreases in absorbance signal and a high value of unfolding midpoints demonstrated the structural integrity and strong resistance to molecular unfolding, thus limiting OxyVita's oxidation within the circulatory system. Song et al. [10] compared vasoactivity and tissue oxygenation resulting from top-load infusions of high molecular weight polymers of OxyVita (oxygenated) and OxyVitaC (COform) using the rat model. Neither product caused vasoconstriction in the rat spinotrapezius muscle in the study, while maintaining tissue oxygenation.

Traumatic Brain Injury (TBI)

Abutarboush et al. [15] undertook the challenge of evaluating the effects of OxyVita on systemic blood pressure and cerebral pial arteriole diameters in healthy rats. The interest of looking into the effects on cerebral pial arterioles was driven by the search for a future therapeutic agent able to safely deliver oxygen to the brain tissue in patients with TBI. Four incremental dosing regiments were used in the study, 2 mg/kg, 25 mg/kg, 50 mg/kg and 100 mg/kg. The control group received saline (0.9%NaCl) and Hextend as a treatment. The measurements of blood pressure were taken at planned intervals, and the sizes of the pial arterioles were observed through intravital microscopy through a cranial window.

The heart rate (HR) did not change significantly during the experiment in the OxyVita group, and increased slightly in the saline group. The study was designed as four, 30-minute infusions, with 10 minute intervals between them. As OxyVita retention time in the circulation is 3 hours, as compared to about an hour or less for saline, the short 10 minutes interval between infusions was not sufficient for the MAP to return to the base line in OxyVita treated animals, and therefore as a result of cumulative effect, the OxyVita group showed an increase in MAP. This is consistent with the previous findings in topload model when an increase in MAP was seen without vasoconstriction in skeletal muscle arterioles [10].

The measurements with intravital microscopy were conducted on small (<50um) and medium (50–100um) pial arterioles, and no vasoconstriction was observed in either of the vessels as a result of injection of four doses/cumulative doses of OxyVita. The injection of vasoconstrictive aqueous barium chloride was performed at the end of the experimental work to confirm proper responsiveness of the arterioles during the experiment.

This study unveiled a very promising future for an application of OxyVita in patients with TBI.

Whole Blood Approach

The main goal of developing oxygen therapeutics was to address the world's critical need for blood transfusion alternatives. The hope of the industry is to develop an artificial blood product with the features of being donor independent, unaffected by contaminations to the blood supply and easy stockpiling for emergency or other clinical indications.

It is recognized that the OxyVita product improves oxygen delivery capacity of blood in in-vitro and animal studies, potentially replacing the function of RBCs. OxyVita can be formulated into a powder form (Fig. 25.1), which is beneficial because it will offer the product with a much longer shelf-life and no strict storage requirements. The powderization process involves the following steps: washing in a buffer solution containing 1.5% of sucrose and trehalose, followed by a treatment with OXYVITA's proprietary molecular protectant, and then lyophilization or spray-drying. The protectant preserves the structural and functional integrity of the components of the powderized hemoglobin polymers, yet allowing for easy reconstitution. Figure 25.2 presents a comparison of the product's Size Exclusion Chromatography before and after spray-drying.

Experimentation with the powderization technology led to its application to other blood components, plasma and platelets. There is an enormous demand for a much longer platelet shelf-life. Platelets generally have only 5 days shelf-life. Previously conducted experiments have shown that platelets collected from a donor and lyophilized on day 4 after collection, using the same methodology as for lyophilizing OxyVita, can be stored at room temperature for 30 days. Thereafter, stored platelet activity was compared to fresh donor platelets, using an aggregometer, PAP-8, with two agonists for the analysis, ADP and collagen [16]. The reconstituted platelets, on day one after the powderization, presented the exact same activity as fresh platelets, and after 30 days of storage at room temperature, their activity decreased by 7% compared to fresh donor platelets. The studies were repeated after 60 days of storage, a decrease of platelet activity by 14% was documented. This data may be extrapolated to lyophilized platelet activity of not less than 58% of fresh platelets, after 6 months.

Previously conducted studies performed combining OxyVita with dried plasma and platelets, utilizing OXYVITA's in-house prepared platelets [16], demonstrated that this coexistence is possible, with no interference, serving as a proof of concept for a possibility of complete blood transfusion alternative product in the future.

Future Direction

Research on OxyVita continues, with many directions being contemplated or explored. Although the main purpose of this product is a safe and effective alternative for the blood transfusion, through the years of research, many other potential applications have arisen. These include applications that are not currently considering a blood transfusion as a primary mode of treatment- such as myocardial infarction (MI), stroke, carbon monoxide poisoning, sickle cell anemia (SCD) - amongst others.

Some of the studies are presented below. Their scope is briefly described.

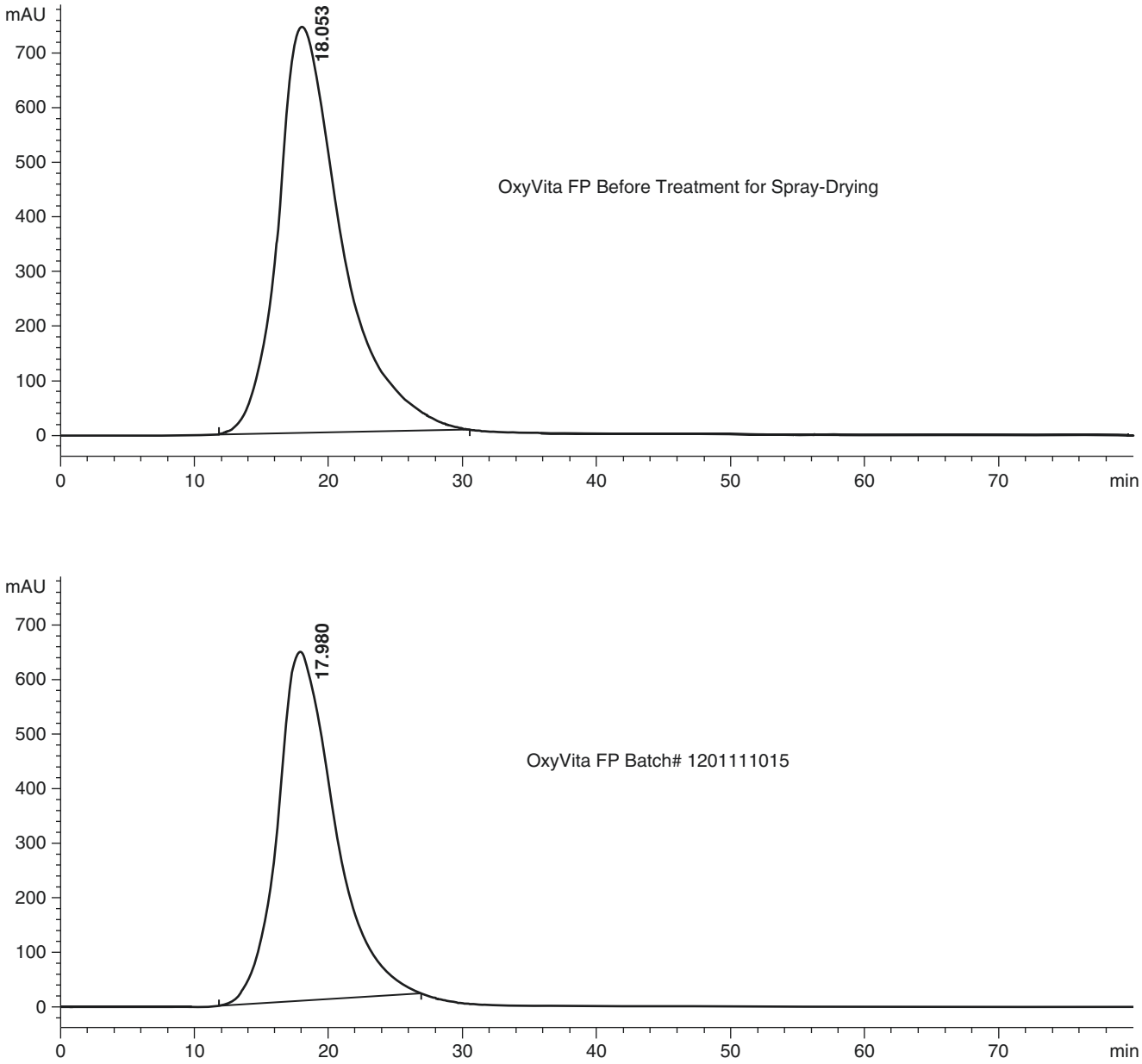


Fig. 25.2 Size Exclusion Chromatography (SEC) analysis: OxyVita liquid and reconstituted powder

Assessment of Effectiveness of OxyVita in the Treatment of Myocardial Infarction and Cardiac Arrest

Ischemic heart disease is the leading cause of death worldwide [17]. According to the statistics provided by the American Heart Association (2019), over 356,000 CAs occur outside the hospital annually, and nearly 90% results in patient mortality [18]. Over 750,000 of CAs occur in the hospital annually with a reported mortality as high as 78% [19, 20].

Based on the limitations of current protocols, specifically the lack of oxygen delivery in the metabolic phase(3rdphase) of CA, HBOCs can be a solution for this unmet need, as they are potentially able to address the ischemia, reperfusion injury, and organ damage resulting from CA.

OxyVita's characteristics make it an ideal candidate. The high oxygen affinity, as noted by Jahr, et al. allows significant oxygen offload to hypoxic tissue in low doses, such as 2-3 ml/kg [21]. This makes it a low volume injection allowing for an insignificant top load and avoiding fluid overload.

Based on the physico-chemical characteristics of the product and its in-vivo behavior [22], as well as the conducted optimization of its parameters for this application, it is hypothesized that early administration of OxyVita during

the first stages of CA, alongside cardiopulmonary resuscitation, can minimize the occurrence of tissue ischemia while avoiding reperfusion injury. The low P_{50} of OxyVita would allow the release of oxygen under conditions of hypoxia only, enabling oxygen delivery to sites vulnerable to ischemia before damage can occur. Tissue perfusion would be optimized, specifically in the metabolic phase, due to OxyVita's ability to avoid vasoconstriction, while allowing for better oxygenation.

Evidence suggests that OxyVita, like other modified hemoglobins, does not lead to activation of complement, once it is introduced as an oxygen carrier, what has substantial implications when considering reperfusion injury [23]. Currently studies are being conducted using Sandwich ELISA with monoclonal antibody to C3a neoepitope [24]. Without complement activation, one of the major pathways of this pathology is greatly hindered, broadening the usage of this treatment option under hypoxic conditions.

Effectiveness of OxyVita in Treatment of Carbon Monoxide (CO) Poisoning

CO poisoning is one of the leading causes of death due to poisoning in the United States. While fire-related smoke inhalation is responsible for most of the cases, non-fire related CO poisoning is still responsible for up to 50,000 emergency department (ED) visits and 1200 deaths per year [25]. CO diffuses rapidly across the pulmonary capillary membrane and binds to the heme moiety of hemoglobin (Hb) with approximately 240 times the affinity of oxygen, causing allosteric changes that significantly diminish the ability of Hb to bind and deliver oxygen to the tissues. Poorly treated CO poisoning can lead to rapidly deteriorating bodily conditions that can result in organ failure and tissue death. Hyperbaric oxygen therapy (HBO) has been shown to have the potential of lowering the mortality rate and preventing late neurocognitive deficits. However, a significant impediment is the limited availability of hyperbaric chambers. Thus, the availability of other methods/products to support oxygen delivery to tissues within the body is essential for the effective treatment of CO poisoning. HBOCs can be a solution for this unmet medical need.

Initial proof-of-concept studies with OxyVita treatment in bench simulation of CO poisoning were already conducted and showed promising results. It is theorized that with the different tertiary and quaternary structure of the OxyVita polymer, its ability to exchange between the CO and oxygen ligands might be totally different than that of the RBCs [26].

OxyVita effectiveness in CO poisoning was investigated by evaluating its oxygen binding capacity and ability to exchange between CO and oxygen ligands over time, at different pH, in various combinations with human blood/tetramers, using UV/Vis spectrophotometry.

As Fig. 25.3 shows, the exchange of CO-O_2 ligands start to occur within the first 5–7 minutes after the addition of the

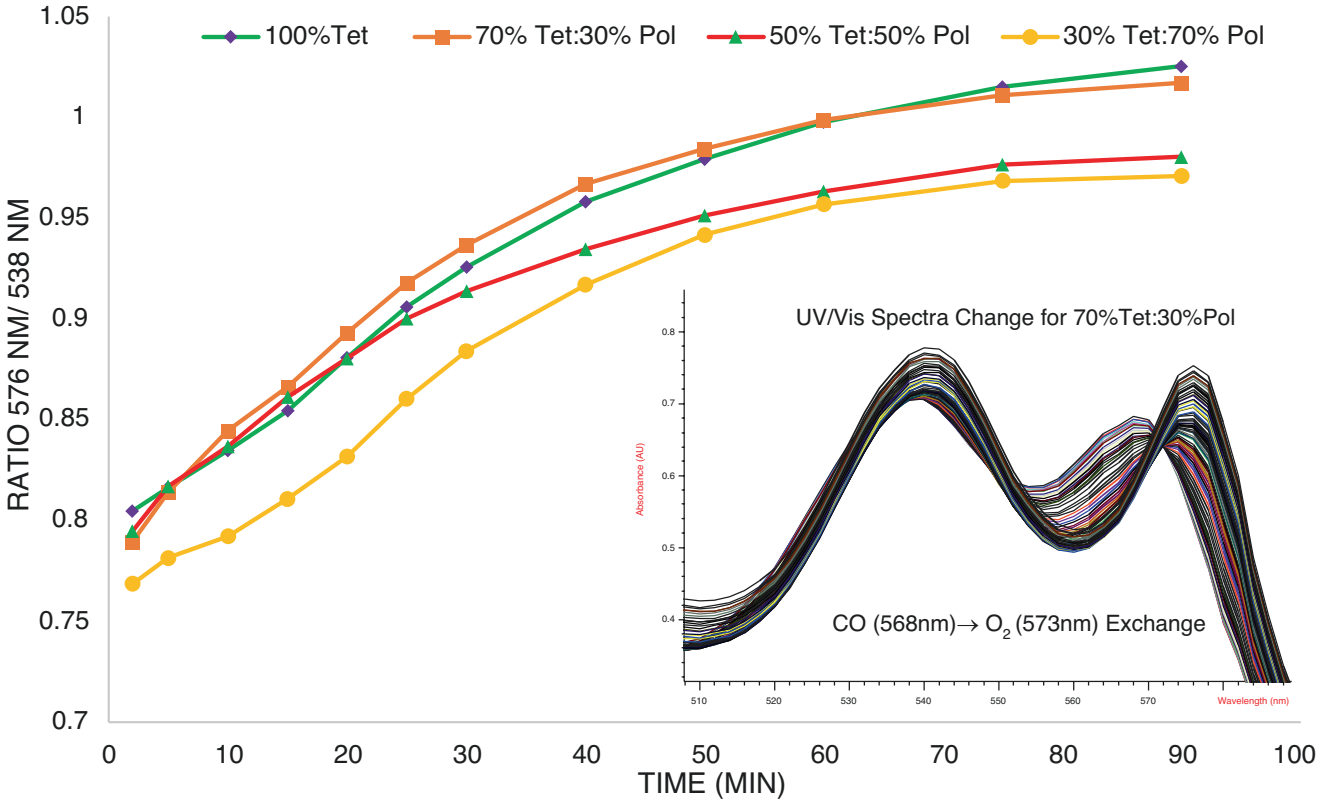


Fig. 25.3 OxyVita and Tetramers: CO and O_2 ligands exchange rate

oxygenated OxyVita to the CO saturated blood/tetramer solution. The solution reaches a good oxygen saturation level at about 15–20 minutes. A combination of 70% blood/tetramers with 30% OxyVita polymer was the most effective for the exchange of CO-O_2 . The study indicated also that the rate of ligand exchange is affected by the pH of the solution (which would translate into the presence of acidosis or alkalosis in human body).

Further experiments are clearly necessary.

Organ Storage Solution

Organ transplantation is a clinically effective, life-saving measure. However, there is a large disparity between the number of donors' organ and the number of individuals on the waiting list. In January 2019, 113,000 individuals were on the waiting list. Regretfully, it is estimated that 20 people will die daily while waiting for a transplant [27]. One of the largest limitations of organ transplants extends from the maximum organ preservation times in which organs are viable from time of procurement to transplantation. This time allotment limits the geographical region for effective organ transplantation and time from organ removal to placement [28].

With the improvements in surgical techniques, aseptic and immunosupressive therapies, the main cause of failure in organ transplant procedures lies still in the condition of the transplanted organ. The longer is the procedure of transplant, including procurement, the worse the condition of the organ, as result of ischemia and hypothermia [29]. Improvement of the organ condition for transplant is seen as a space for improvement in the organ transplant field.

The most possible way to achieve this goal seems to be a formulation of an organ storage solution with an oxygen carrying capacity. This would allow the organ to maintain aerobic metabolism outside of the human body and prevent anaerobic metabolism, an eventuallity, which leads to cell dysfunction and death. The application of an organ storage/preservation solution could allow for the extension of storage time outside the body, and therefore an extended time to match, transport, and expand the geographic limitation.The betterment of the organ's condition would result in a greater successs of transplant surgeries.

It would seem to be essential to have an oxygen carreier as a component of the organ storage solution to maintain oxygen delivery. This contrasts previously attempted techniques such as oxygenation of a perfusion solution/and or gaseous oxygen pillow over the transported organ. Excessive amounts of oxygen in the environment of the stored organ contributes to the formation of free oxygen, which creates an avalanche of oxygen radicals (ROS) [30]. These radicals can lead to deterioration of the cell walls, disruption of ion equilibrium, changes of osmolarity, acidosis, and eventually, cell death. Only through delivery of the oxygen into microcirculation, the aerobic metabolism can be maintained and stable organ conditions can be achieved, leading to a higher possibility of survival of the transplanted organ tissue.

Proof of concept preliminary studies conducted at the UWM School of Medicine, in Olsztyn, Poland, evaluated the role of OxyVita as a component of an organ storage solution to preserve renal tissue in a rabbit model (material in preparation for publication).

Further studies are currently in progress on animal kidneys, aiming to assess the organ condition after 24–48 hours of storage in the storage solution with OxyVita, by analyzing serum chemistry and biomarkers, urine output and urine biochemical analyses, sonographic techniques for a real-time assessment of condition of stored organ's tissue and histopathology analysis by sampling during and after the storage procedure.

The Effect of OxyVita Hb on Erythrocytes Sickling in Patients with Sickle Cell Disease (SCD)

Millions of people worldwide are afflicted with SCD, a hereditary life-threatening genetic blood disorder, characterized by a point mutation in β-globin gene (HBB), leading to production of sickle hemoglobin (HbS). HbS polymerizes when deoxygenated [31, 32]. An HBOC which is capable of being used in a top-load application could serve as a mode of treatment when approaching/ or in the crisis state.

Due to the reduced oxygen-carrying capacity of HbS in SCD patients, preventing hypoxia by oxygen therapy has shown to be beneficial. Studies, both in vitro and in vivo, have shown that oxygen by itself may act as a potent anti-sickling agent, and works by preventing and reversing erythrocytes sickling in SCD [33]. However, the use of oxygen therapy still remains controversial because high levels of oxygen are linked to the suppression of erythropoietin, a key cytokine secreted by the kidneys to promote erythrocyte production. Oxygen therapy is, therefore, only recommended when oxygen levels drop below a critical threshold.

The analysis of Alayash et all [34] points the specific requirements for the characteristics of the oxygen carrier to be able to function effectively as the treatment for SCD. OxyVita fits the pointed requirements, like lack of NO scavenging, low P_{50} and resistance to heme-mediated oxidative reaction. Therefore, it should be a good fit for the treatment of SCD and currently undertaken study aims to determine whether, and the dose at which, OxyVita Hb, is able to delay in vitro HbS polymerization and decrease reversible sickled erythrocyte sickling.

In the current study, the focus is on the polymerization process of HbS itself. Previous studies have shown that the

amount of intracellular deoxygenated HbS polymer increases monotonically with decreasing oxygen saturation [35]. During the early stage of oxygen dependent HbS polymerization, the process of sickled red blood cell formation is reversible. The reversibly sickled erythrocytes (RSC) are able to return to the normal biconcave shape upon oxygen binding and prevent subsequent progressing to irreversibly sickled erythrocytes (ISC). OxyVita, with its small molecular size (MW = 17 MDa) and its oxygen-carrying capacity (P_{50} = 6.4 mmHg) is potentially capable of reaching the microvasculature and delivering oxygen to reverse sickling [36].

Currently planned study aims to: (1) Measure the protective effect of various concentrations of OxyVita on in vitro polymerization. (2) Measure the ability of OxyVita to assist in the reversal of RBC sickling in vitro. (3) Test the hypothesis that OxyVita slows down the creation of irreversible sickled RBCs.

After several circles of deoxygenation and HbS polymerization, a fraction of circulating erythrocytes is permanently deformed into a sickle shape even after vigorous oxygenation [35]; these are defined as irreversibly sickled cells (ISC). ISC are dense, dehydrated, and viscous with a low affinity for oxygen and a very short life span [37]. The short-lived, rigid ISCs make major contributions to the hemolytic events/vaso-occlusive crises in SCD patients. It therefore becomes a therapeutic interest to investigate approaches that may significantly slow down the progress of formation of ISC. The mechanisms proposed for the progress of RSC to ISC are due to the change of erythrocyte membrane properties. The decrease in blood oxygen partial pressure and the polymerized deoxygenated HbS activate a membrane channel called P_{sickle}. Open P_{sickle} channels allow the influx of Ca^{2+} and activation of the Gardos channels. The activation of Gardos channels eventually causes efflux of K^+ and Cl^-, followed by water loss and RBC dehydration. OxyVita Hb as an oxygen carrier, is able to increases the oxygen saturation and partial pressure of the arterial blood and inhibit the activation of P_{sickle} channels and Ca^{2+} influx. A protocol is in the works to test the effect of OxyVita on the ratio of RSC/ISC.

ROS Induced Oxidative Stress: Blood Transfusion Versus Blood Substitutes

Overproduction of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide, results in oxidative stress, which leads to cellular damage. RBCs have an inherent ability to combat oxidative stress through enzymatic (peroxiredoxin-2, catalase, superoxide dismutase, and glutathione peroxidase) and non-enzymatic (glutathione, vitamins C and E, and urate) antioxidant pathways. Previous studies provided evidence that donated RBCs, during blood processing and storage, will face the environmental challenges like lower temperature, differing pH and residual plasma, and imbalance between oxidants and antioxidants [38]. RBCs begin to accumulate oxidative lesions due to damages to its lipid and protein structures. Such alterations of RBC components may contribute to cell lysis with releasing vesicular microparticles including free hemoglobin, heme, and iron, which may contribute to the generation of additional ROS. It has been suggested that since these microparticles are abundant in iron, the free radicals are generated through iron dependent mechanisms - the Haber-Weiss and Fenton reactions [39].

Further, in diseases requiring chronic transfusions (like β-Thalassemia, myelodysplastic syndrome), significant iron loading leads to free iron exceeding the binding capacity of the plasma transferrin, and therefore, results in an increase in non-transferrin bound iron (NTBI). NTBI promotes the production of free hydroxyl radicals and the accumulation of transfusion-associated iron in tissues causing organ damages [40]. In addition to causing parenchymal organ damage by ROS via upregulation of biological cellular processes that increase cellular apoptosis, iron overload by repeated blood transfusions also causes dysregulation of the hematopoietic system, most probably by the suppressive effect on hematopoiesis. This includes an increase in apoptosis of hematopoietic progenitors, shortened life spans of mature RBCs, and inhibited differentiation of hematopoietic stem cells [41]. To alleviate this problem, iron chelating agents such as deferoxamine are introduced in iron overloaded environments. Deferoxamine has been shown to partially reduce iron overload and injury and block certain ROS-related signaling pathways. It is believed that the iron chelating agent serves as an antioxidant by eliminating free-iron species [42].

In an effort directed towards a safe and efficacious replacement for blood transfusion/RBCs transfusion, the ROS generation during the storage of HBOCs has to be taken into consideration. This assessment will also serve as a safety characteristic for clinical use of the blood substitutes. Moreover, the research should be extended to investigation of the benefits of usage of oxygen carrier treatment in repeated blood transfusion with the addition of antioxidants, such as deferoxamine as an adjunct therapy.

Previous investigations also showed that OxyVita has a greater tendency to resist molecular unfolding when compared to blood with tetramer hemoglobins, and therefore, OxyVita does not readily release free heme-iron into the circulatory system [8]. In the current study, we compare the level of ROS formation in the stored OxyVita product with the one of stored RBCs, the ROS levels after the introduction of ROS formation promoting agents, as well as after the addition of antioxidants, to mitigate the ROS damage, in order to conclude on the possibility of limiting/controlling the ROS formation process.

Summary

OxyVita, as a new generation of hemoglobin-based oxygen carriers, has had the benefit of avoiding the shortcomings of previous generations of products. The polymer has proven both by in-vitro and in-vivo animal studies, to have promises in many different therapeutic areas – many of which have not been considered to be a potential area for blood use. The characteristics of the product, as well as the ability to customize the molecule as necessary (size, P_{50}, etc), have granted a unique product that can fit many different applications.

Over time, the product has been shaped by the teams of researchers that have worked with it, independently, as well as collaboratively, with its creators. The future of this product is still being decided, as well as the direction that it will ultimately take. With potential for use in both human and animal markets, spanning from blood transfusion alternative to other unmet and previously unconsidered applications, there seems to be a multitude of avenues for the future. It is the hope of the teams that have worked with it that the product will progress to take a position to support and improve the healthcare industry in the coming years.

Key Points

- OxyVita is a new generation oxygen carrier, a polymeric hemoglobin manufactured utilizing zero-link polymerization technology, with very defined and specifically designed characteristics.
- Extensive in-vitro and in-vivo testing of OxyVita has brought significant insights into the physico-chemical characteristics of the product and its mechanisms of action, which translates into the product's functional attributes and potential physiologic applications.
- Research on the product continues to assess the full scope of the product's safety profile in applications as a human and animal therapeutic.
- The product's aim is to be a safe and effective alternative for the RBC/blood transfusion in the future.
- Other product applications are being considered for conditions currently treated with blood transfusions as well as for these in which this type of treatment is not considered as a primary mode of therapeutic intervention.

References

1. Harrington JP, Wollocko H. Zero-link hemoglobin (OxyVita): impact of molecular design characteristics on pre-clinical studies. In: Textbook – hemoglobin based oxygen carriers and red cell substitutes and oxygen therapeutics. Springer; 2013. p. 283–97.
2. Harrington JP, Wollocko J, Kostecki E, et al. Physicochemical characteristics of OxyVita hemoglobin, a zero-linked polymer: liquid and powder preparations. Artif Cells Blood Substit Biotechnol. 2011;39:12–8.
3. Grabarek Z, Gergely J. Zero-length crosslinking procedure with the use of active esters. Analyt Biochem. 1990;185:131–5.
4. Staros JV, Wright RW. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. Analyt Biochem. 1986;156:220–2.
5. Razynska A, Bucci E. Zero-link polymerization: a new class of polymeric hemoglobins. In: Blood substitutes- present and future perspectives; 1998. p. 265–79.
6. Bucci E, Kwansa H, Koehler RC, Matheson B. Development of zero-link polymers of hemoglobin, which do not extravasate and do not induce pressure increases upon infusion. Artif Cells Blood Substit Immobil Biotechnol. 2007;35(1):11–8. https://doi.org/10.1080/10731190600974277.
7. Harington JP, Orlig K, Zito SL, et al. Structural and Redox Behavior of OxyVita Hb, a zero-linked polymeric hemoglobin: comparison with natural acellular polymeric hemoglobins. Artif Cells Blood Substit Biotechnol. 2010;38:64–8.
8. Wollocko H, Anvery S, Wollocko J, et al. Zero-link polymerized hemoglobin (OxyVita®Hb) stabilizes the heme environment: potential for lowering vascular oxidative stress. Artif Cells Nanomedicine Biotechnol. 2017;45(4):701–9.
9. Matheson B, Kwansa HE, Bucci E, et al. Vascular response to infusions of a nonextravasating hemoglobin polymer. J Appl Physiol. 2002;93:1479–86.
10. Song BK, Nugent WH, Moon-Massat PF, et al. Effects of top-loading a zero-link bovine hemoglobin (ZL-HbBv), OxyVita™, on systemic and microcirculatory variables. Mil Med. 2012;178(5):570–7.
11. Mito T, Nemoto M, Kwansa H, et al. Decreased damage from transient focal cerebral ischemia by transfusion of zero-linked hemoglobin polymers in mouse. Stroke. 2009;40:278–84.
12. Rebel A, Ulatowski JA, Kwansa H, et al. Cerebrovascular response to decreased hematocrit: effect of cell-free hemoglobin, plasma viscosity, and CO_2. Am J Physiol Heart Circulat Physiol. 2003;285:1600–8.
13. Reynolds PS, Barbee RW, Skaflen MD, et al. Low-volume resuscitation cocktail extends survival after severe hemorrhagic shock. Shock. 2007;28:45–52.
14. Jahr JS, Weeks DL, Desai P, et al. Does OxyVita, a new-generation hemoglobin-based-oxygen carrier, or Oxyglobin acutely interfere with coagulation compared with normal saline or 6% Hetastarch? A ex-vivo thromboelastography study. J Cardiothoracic Vascular Anesthesia. 2008;22:34–9.
15. Abutarboush R, Aligbe C, Pappas G, et al. Effects of the oxygen-carrying solution OxyVita C on the cerebral microcirculation and systemic blood pressures in healthy rats. J Funct Biomater. 2014;5:246–58. https://doi.org/10.3390/jfb5040246.
16. Wollocko H, Grzegorzewski W, Smyk L, et al. OxyVita®C, a next-generation Haemoglobin-based oxygen carrier, with coagulation capacity (OVCCC). Modified Lyophilization/spray-drying process: proteins protection. Artif Cells Nanomedicine Biotechnol. 2017;45(7):1350–5. https://doi.org/10.1080/21691401.2017.1339052.
17. Pagidipati NJ, Gaziano TA. Estimating deaths from cardiovascular disease: a review of global methodologies of mortality measurement. Circulation. 2013;127(6):749–56. https://doi.org/10.1161/CIRCULATIONAHA.112.128413.
18. Benjamin EJ, Muntner P, Bittencourt MS. Heart disease and stroke statistics-2019 update: a report from the American Heart Association. Circulation. 2019;2019, 139(10).
19. Adams BD, et al. Cardiac arrests of hospital staff and visitors: experience from the national registry of cardiopulmonary resuscitation. Resuscitation. 80(1):65–8.
20. Kleinman ME, Brennan EE, Goldberger ZD, et al. Part 5: adult basic life support and cardiopulmonary resuscitation quality: 2015 American Heart Association guidelines update for cardiopulmo-

nary resuscitation and emergency cardiovascular care. Circulation. 2015;132(suppl2):S414–35.
21. Jahr JS, Nesargi SB, Lewis K, et al. Blood substitutes and oxygen therapeutics: an overview and current status. Am J Ther. 2002;9:437–43.
22. Harrington JP, Wollocko H. Pre-clinical studies using OxyVita hemoglobin, a zero-linked polymeric hemoglobin: a review. J Artif Organs. 2010;13:183–8.
23. Ning J, Chang MS. Effects of homologous and heterologous stroma-free hemoglobin and polyhemoglobin on complement activation, leucocytes and platelets. In: Biomaterials, Artificial cells & Artificial Organs | Published online. Taylor & Francis; 2009. p. 219–32. https://doi.org/10.3109/10731199009117303.
24. Zilow G, Naser W, Rutz R, Burger R. Quantitation of the anaphylatoxin C3a in the presence of C3 by a novel sandwich ELISA using monoclonal antibody to a C3a neoepitope. J Immunol Methods. 1989;121:261–8.
25. Clardy PF, Manaker S, Perry H. Carbon monoxide poisoning. https://www.uptodate.com/contents/carbon-monoxide-poisoning. Published 2018. Accessed 20 Jan, 2019.
26. Harrington JP, Wollocko J. Ligand exchange: increased structural and redox stability for zero-linked polymeric OxyVita hemoglobin, a hemoglobin-based-oxygen-carrier. Biophysical J. 2011;100(3 Supplement 1):220A. https://doi.org/10.1016/j.bpj.2010.12.1414.
27. United States Department of Health and Human Services. Organ Donation Statistics. https://www.organdonor.gov/statistics-stories/statistics.html. Published 2019. Accessed 31 March, 2019.
28. Organ Procurement And Transplantation Network. How organ allocation works. https://optn.transplant.hrsa.gov/learn/about-transplantation/how-organ-allocation-works/. Published 2019. Accessed 31 March, 2019.
29. DuBose TD Jr. Hyperkalemic hyperchloremic metabolic acidosis: Pathophysiologic insights. PlumX Metrics. 1997;51(2):591–602. https://doi.org/10.1038/ki.1997.85.
30. Jia Y, Alayash A. Effects of cross-linking and zero-link polymerization on oxygen transport and redox chemistry of bovine hemoglobin. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism. 2009;1794(8):1234–42. https://doi.org/10.1016/j.bbapap.2009.04.008.
31. Piel FB, Steinberg MH, Rees DC. Sickle cell disease. N Engl J Med. 2017;377(3):305.
32. Sundd P, Gladwin MT, Novelli EM. Pathophysiology of sickle cell disease. Annu Rev Pathol. 2019;14:263–92.
33. Zipursky A. Oxygen therapy in sickle cell disease. Am J Pediatr Hematol Oncol. 1992;14(3):222–8.
34. Alayash AI. Hemoglobin-based blood substitutes and the treatment of sickle cell disease: more harm than help? Biomolecules. 2017;7(1).
35. William AE, Franklin B. Treating sickle cell disease by targeting HbS polymerization. Proc Natl Acad Sci U S A. 1980;77(9):5487–91.9.
36. Jia Y, Alayash AI. Effects of cross-linking and zero-link polymerization on oxygen transport and redox chemistry of bovine hemoglobin. Biochim Biophys Acta. 2009;1794(8):1234–42.
37. Noguchi CT, Torchia DA, Schechter AN. Determination of deoxyhemoglobin S polymer in sickle erythrocytes upon deoxygenation. EBioMedicine. 2015;2(11):1669–76.
38. Bardyn M, Tissot JD, Prudent M. Oxidative stress and antioxidant defenses during blood processing and storage of erythrocyte concentrates. Transfus Clin Biol. 2017;25(1):96–100.
39. Neal MD, Raval JS, Triulzi DJ, et al. Innate immune activation after transfusion of red blood cells. Transfus Med Rev. 2013;27(2):113–8.
40. Ozment CP, Turi JL. Iron overload following red blood cell transfusion and its impact on disease severity. Biochim Biophys Acta. 2009;1790(7):694–701.
41. Chai X, Li D, Cao X, et al. ROS-mediated iron overload injures the hematopoiesis of bone marrow by damaging hematopoietic stem/progenitor cells in mice. Sci Rep. 2015;5(10181):1–11.
42. Lu W, Zhao M, Rajbhandary S, et al. Free iron catalyzes oxidative damage to hematopoietic cells/mesenchymal stem cells *in vitro* and suppresses hematopoiesis in iron overload patients. Eur J Haematol. 2013;91(3):249–61.

Lumbricus terrestris Erythrocruorin: A Novel Blood Substitute from a Terrestrial Earthworm

26

Sean Dowd and Jacob Elmer

Introduction

While most mammals have hemoglobins inside of red blood cells (RBC), annelids have an especially unique type of hemoglobin that is called erythrocruorin (Ec). Ecs have several attractive properties that make them attractive as RBC substitutes, including a relatively high molecular weight (MW) and a resistance to oxidation and NO scavenging [1–3]. This chapter will describe studies on the Ec of the terrestrial earthworm *Lumbricus terrestris* (LtEc), while Chap. 34 is focused on the Ec of the marine lugworm, *Arenicola marina* (Hemarina).

Macromolecular Structure of LtEc

Assembly of the Hexagonal Bilayer

The relatively large size of LtEc (~3.6 MDa) has inspired over a century of research on its composition and assembly. Indeed, Ecs from earthworms were among the first proteins to be crystallized in 1840 and a key focus of Svedberg's initial ultracentrifugation studies in 1933. LtEc was also one of the first proteins to be studied with electron microscopy [4–8], but a high resolution (3.5 Å) crystal structure (PDB ID: 2GTL) for LtEc was not available until 2006 [8].

The crystal structure of LtEc revealed that it is a macromolecular assembly of 144 globin units and 36 linker proteins [8–10]. Unlike tetrameric mammalian hemoglobins (e.g., $\alpha_2\beta_2$), LtEc may contain up to 6 different globin subunits (A, B, C, D1, D'1 and D2) [8, 9, 11, 12] that assemble into ABCD tetramers. It is worth noting that the three types of D subunits share a high degree of sequence similarity. Indeed, D'1 is only a slight variant of D1 with threonine instead of alanine at position 84, while D1 and D2 differ by 28 amino acids [12]. Consequently, any of the D subunits may be used to form an ABCD tetramer, but D1 is more prevalent than D2 in ABCD tetramers [11, 12].

After the globins assemble into tetramers, three tetramers associate to form a dodecamer ($A_3B_3C_3D_3$). This dodecamer is then bound by a trimer of linker proteins (L1, L2, L3, and/or L4) to form a protomer. The stoichiometry of these linkers in native LtEc is roughly L1:L2:L3:L4 = 1:1:1:0.5 [9, 13], but the linker trimer can also be assembled *in vitro* with only a binary mixture of L1 + L3 or L2 + L4. Finally, 12 of the linker/globin protomers assemble through interactions between coiled-coil domains in the linker trimers to form a hexagonal bilayer (HBL; 6 protomers/layer) with D_6 symmetry, a MW of approximately 3600 kDa, and a diameter of 30 nm (see Fig. 26.1 and Table 26.1) [8–10].

Some studies have reported molecular weights for native LtEc that are higher than 3.6 MDa. These higher MWs may be due to the presence of an additional dodecamer that binds to the linkers in the central toroid of the HBL, but this phenomenon has only been observed with LtEc in a single study and only in a small fraction of HBLs under specific conditions [15]. Alternatively, it has also been reported that LtEc HBLs can dimerize at high concentrations [17]. In either case, the MW of fully assembled LtEc is significantly larger than tetrameric human adult Hb (HbA), which has a MW of 64 kDa and a diameter of 5 nm [9, 18].

Structural Stability of LtEc

Close examination of the LtEc structure reveals that the protomers in each bilayer are "staggered," thereby allowing the coiled-coils of the linker trimers to intercalate and form a more compact structure (also known as a Type I HBL) that maximizes subunit contacts to stabilize the HBL and prevent dissociation. This is in contrast to other Ecs with protomers that are "eclipsed" (i.e., Type II HBL), like the Ec of *Arenicola marina* (AmEc) (see Chap. 33) [19, 20].

S. Dowd · J. Elmer (✉)
Department of Chemical and Biological Engineering, Villanova University, Villanova, PA, USA
e-mail: sdowd5@villanova.edu; jacob.elmer@villanova.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_26

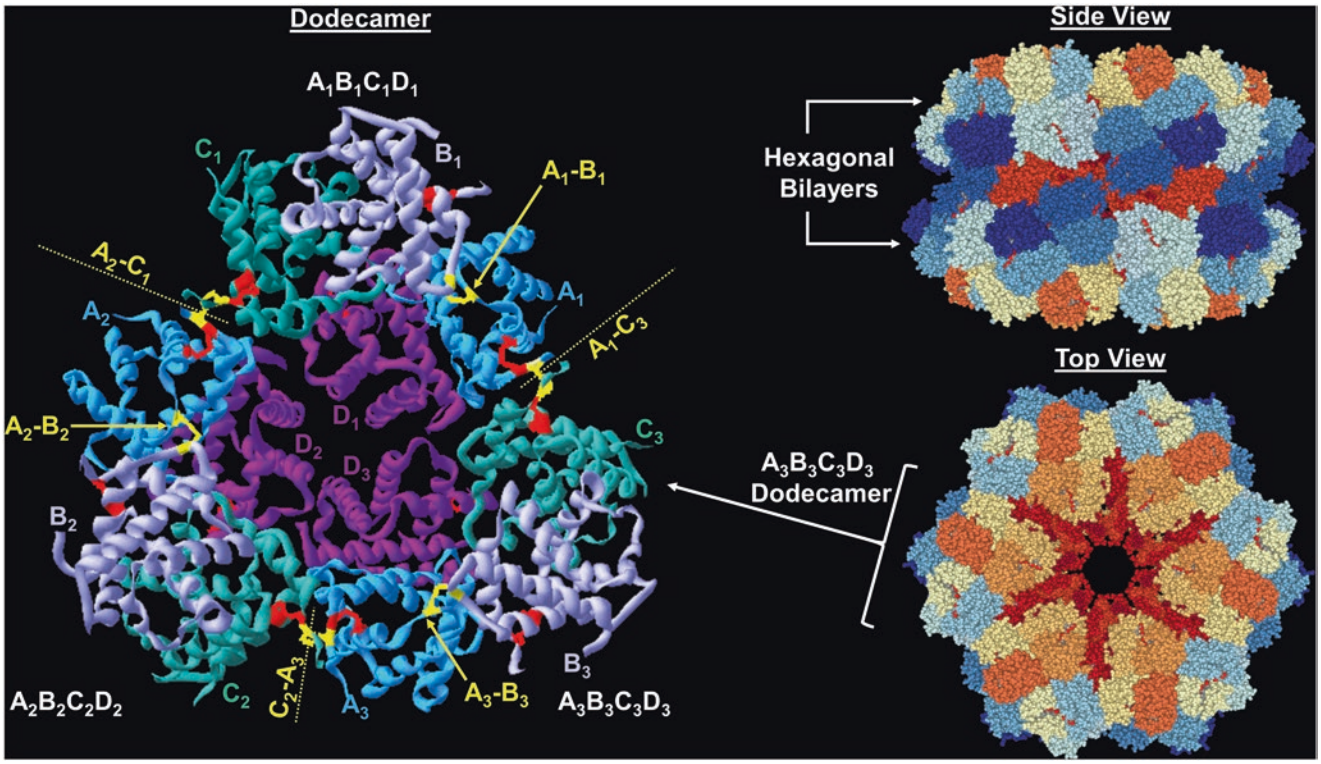


Fig. 26.1 Structure of LtEc (PDB ID 2GTL). Left: Structure of the $A_3B_3C_3D_3$ dodecamer with ABCD tetramer interfaces denoted as yellow dashed lines, while intramolecular and intermolecular disulfide bonds are highlighted in red and yellow, respectively. (prepared with Swiss PDB Viewer) Right: Side and top views of the complete hexagonal bilayer (HBL) structure of LtEc (180 subunits, 3.6 MDa). Linkers in the central toroid are colored red and surrounded by multicolored globin subunits (prepared with NGL Viewer [14])

Table 26.1 Biophysical properties of LtEc and HbA [9, 15, 16]

	MW (kDa)	Diameter (nm)	Globin Subunits	O_2 Affinity (P_{50}; mmHg)	Cooperativity @ pH 7.4
HbA	64	5	4	11	2.7
LtEc	3600	30	144–156	28	3.7

MW molecular weight, *kDa* kilodalton, *HbA* adult hemoglobin

In addition to linker proteins, there are also several disulfide bonds in LtEc that increase its stability. For example, each globin has an intramolecular disulfide bond (highlighted red in Fig. 26.1) that contributes to the thermal stability of each globin. Additional intermolecular disulfide bonds also form between the A and B subunits of each tetramer and between the A and C subunits of adjacent tetramers to prevent dissociation of the dodecamers [9]. Furthermore, each linker subunit is also stabilized by up to 5 intramolecular disulfide bonds within their low density lipoprotein (LDL) receptor-like domains, which are responsible for binding the $A_3B_3C_3D_3$ dodecamer to form the protomer [8, 9].

In addition to disulfides, LtEc also has several Ca^{2+}, Zn^{2+}, and Cu^{2+} binding sites that contribute to its stability [21]. Interestingly, the Zn^{2+} and Cu^{2+} metal binding sites possess some limited superoxide dismutase (SOD) activity that may partially protect LtEc from oxidation [22, 23]. Meanwhile, Ca^{2+} is required for LtEc to properly fold and decreases its dissociation in alkaline environments [23–25]. Calcium also increases the oxygen-binding affinity and cooperativity of LtEc [23].

Overall, this combination of disulfide bonds, metal binding sites, and other structural features makes native LtEc much more resistant to subunit dissociation and denaturation than HbA, even at high temperatures or concentrations of urea [25–27]. This high stability is an attractive property for a RBC substitute since it prevents extravasation while also prolonging the circulation half-life of LtEc.

Glycosylation

ESI-MS analysis of individual subunits isolated from native LtEc revealed that the A and L1 subunits can be glycosylated [11, 13]. Specifically, these subunits appear to be mannosylated with $(GlcNAc)_2(Man)_n$ glycans, where n = 6–9 for the A subunit and n = 8–9 for the L1 subunit [11]. Similar glycosylation patterns have also been observed in the hemoglobins of marine annelids (e.g., *Perineries aibuhitensis, Riftia pachyptila*, and *Lamellibrachia satsuma*) and it has been suggested that these glycans may contribute to the stability of the HBL [28–32].

The cDNA sequence for the L1 linker in Fig. 26.2 shows that glycosylation should occur at Asn118 (glycosylation site = N-X-S/T = NIT for L1). Another potential glycosyl-

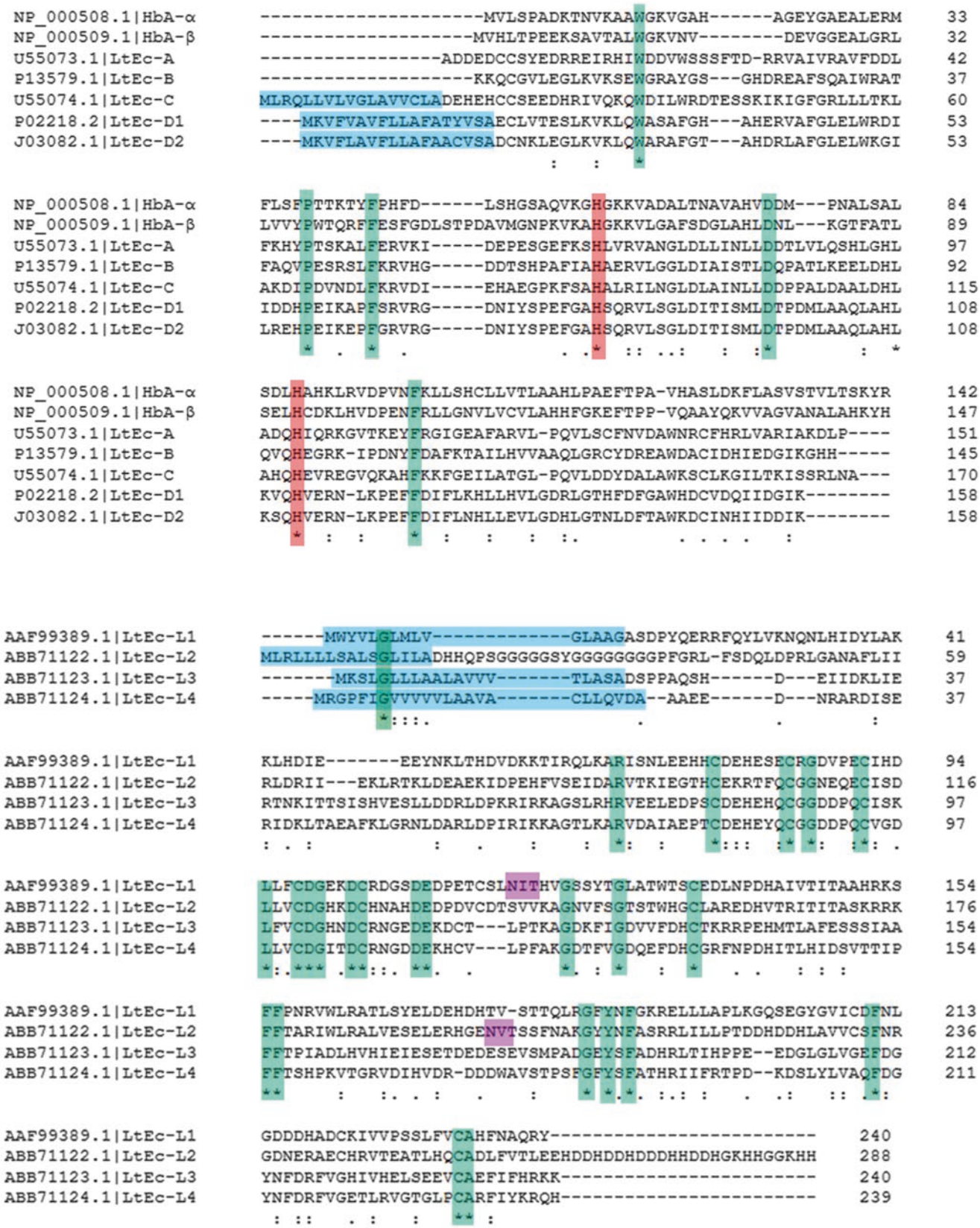


Fig. 26.2 Amino acid sequences of LtEc subunits. Top: Alignment of LtEc and human hemoglobin globin sequences. Conserved amino acids are highlighted green, while proximal and distal histidines are highlighted red, and secretion tags are highlighted blue. Bottom: Alignment of LtEc linkers sequences, with potential glycosylation sites highlighted in purple. Alignments prepared with Clustal Omega [33]

ation site also exists at Asn198 (NVT) in the L2 linker chain, but glycosylation of L2 has not yet been reported. The glycosylation site on the A globin is unclear, since the cDNA sequence for the A and B globins has not yet been determined. The partial sequences of these globins have been estimated with X-ray crystallography (see Fig. 26.2), but those sequences may be incomplete since they lack secretion tags (which are present on all other globins and linkers) and no potential glycosylation site is observed in the A subunit [12, 24, 34–36]. However, while it was not mentioned by the authors that conducted the X-ray crystallography study, it is possible that there is a glycosylation site at position 85 in the native A subunit (e.g., NDT instead of DDT), but that asparagine may have been converted to an aspartate in the process of removing glycans to in preparation for X-ray crystallography.

Oxygen Transport and Interactions with NO

Oxygen Affinity and Cooperativity

Another interesting difference between HbA and LtEc is the oxygen affinity of the two isolated hemoglobins. While human RBCs have a relatively low oxygen affinity (P_{50} = 26 mmHg), purification of HbA from the RBCs removes the allosteric cofactor 2,3-DPG and significantly increases the oxygen affinity of pure HbA (P_{50} = 11 mmHg) [9, 37]. In contrast, the oxygen affinity of purified LtEc (P_{50} = 28 mmHg) is similar to RBCs and it is not influenced by 2,3-DPG [9, 38]. Instead, the allosteric effector of LtEc is Ca^{2+}, which is available in the bloodstream and increases the O_2 affinity of LtEc. For example, the O_2 affinity of LtEc doubles in 170 mM Ca^{2+} compared to 12.4 mM Ca^{2+} [39]. Other divalent cations, like Ba^{2+}, Sr^{2+}, and Mg^{2+}, also have a similar effect on the O_2 affinity of LtEc [9].

It is also interesting to note that isolated dodecamers and ABCD tetramers have O_2 affinities that are similar to native LtEc [9, 40]. The isolated ABC trimer and D monomer, however, have higher O_2 affinities [9, 40]. Finally, while the Hill coefficient (a measure of cooperativity) of HbA and RBCs is approximately 2.5–2.7 under physiological conditions, it is slightly higher for LtEc (n = 3.7) [9, 41]. This increase in cooperativity reflects the increased number of subunit interactions within the LtEc dodecamer [9].

The similarity in P_{50} and n values between LtEc and RBCs suggests that LtEc should transport O_2 in a fashion similar to whole blood and effectively transport enough O_2 from the lungs to the tissues. Indeed, multiple studies have shown that exchange transfusions of LtEc can effectively oxygenate tissues in hamsters without any adverse effects [42–44].

Heme Oxidation

Erythrocruorins, hemoglobins, and RBCs are all vulnerable to oxidation of the heme iron, a process that negates oxygen transport and generates harmful reactive oxygen species that can lead to significant oxidative stress *in vivo* [45]. Furthermore, even low levels of LtEc oxidation (e.g., 15–25%) can also induce the dissociation of the D subunit from the ABC trimer [46].

However, LtEc has some unique properties that allow it to resist oxidation. First of all, the A, B, and D globins have bulky aromatic residues at position B10 that penetrate the heme pocket and stabilize the bound oxygen to prevent its escape as superoxide (O_2^-) [47]. Indeed, mutating myoglobin and human hemoglobin to introduce similar bulky residues at the same B10 position decreases oxidation rates relative to the wild type globins [48–50]. LtEc also has a positive redox potential (+112 mV), which means that it can be reduced with reducing agents like ascorbic acid [26]. In contrast, human hemoglobin and horse myoglobin have negative redox potentials (−50 mV and −157 mV, respectively) that reflect an increased susceptibility to oxidation [51, 52]. Indeed, while some mammalian HBOCs have been reported to rapidly oxidize [53], our preliminary pharmacokinetic studies with LtEc in hamsters have shown no increase in oxidation up to 48 hours (Fig. 26.3) [43]. This resistance to oxidation is very important for a RBC substitute since it prevents oxidative stress while ensuring oxygen delivery to the patient's tissues.

Another important concern is the oxidation of LtEc during storage at elevated temperatures [46, 54, 55]. Ideally, any HBOC should be able to resist oxidation at high temperatures for an extended period to enable its use in remote areas and battlefield scenarios. Fortunately, our previous studies have shown that the oxidation rate of LtEc during storage can be significantly decreased or eliminated by dissolving it in a modified Ringer's Lactate solution or deoxygenating the LtEc solution [54, 55]. However, if oxidation were to occur during storage, we have also shown that ascorbic acid can fully reduce oxidized LtEc [55]. Since ascorbic acid exists in human blood, oxidized LtEc may also be reduced following transfusion.

Nitric Oxide

One of the most undesirable side effects observed with mammalian HBOCs is nitric oxide (NO) dioxygenation, a reaction in which NO in the bloodstream reacts with heme-bound oxygen to simultaneously form nitrate (NO_3^-) and oxidize the heme iron (Fe^{2+} to Fe^{3+}). Depletion of NO by this reaction causes severe adverse effects *in*

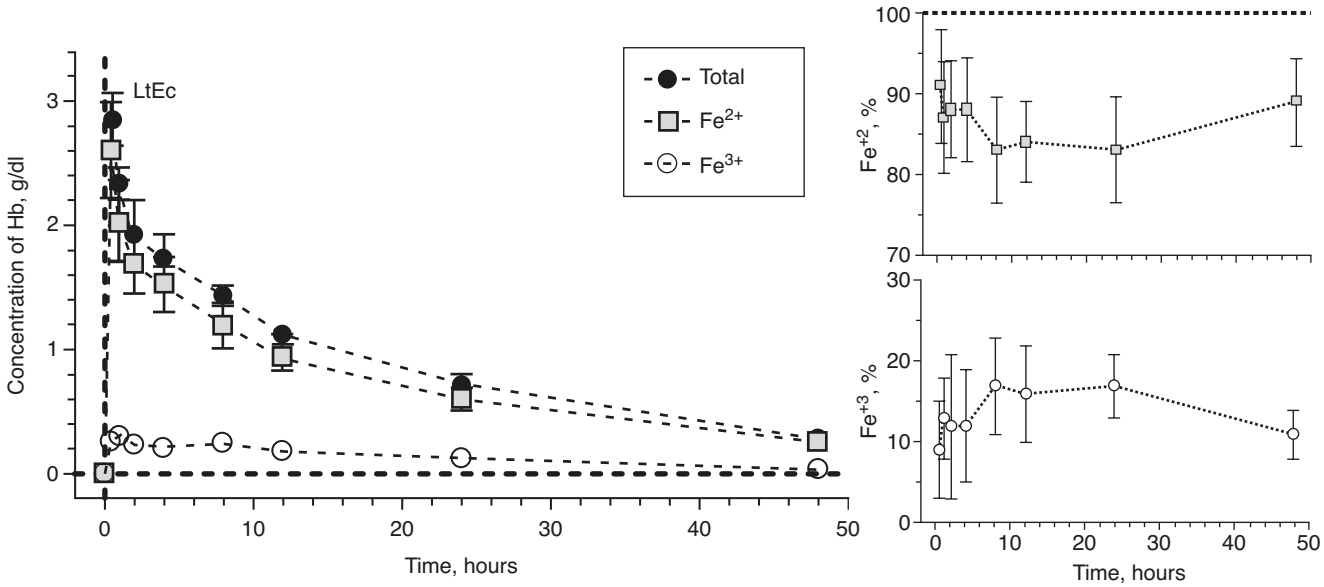


Fig. 26.3 Pharmacokinetic analysis of LtEc following exchange transfusions in hamsters. Left: Clearance of LtEc (Hb) following an initial transfusion of 3 g/dL LtEc. Right: Levels of reduced (Fe^{2+}) and oxidized (Fe^{3+}) LtEc observed up to 48 hours following transfusion

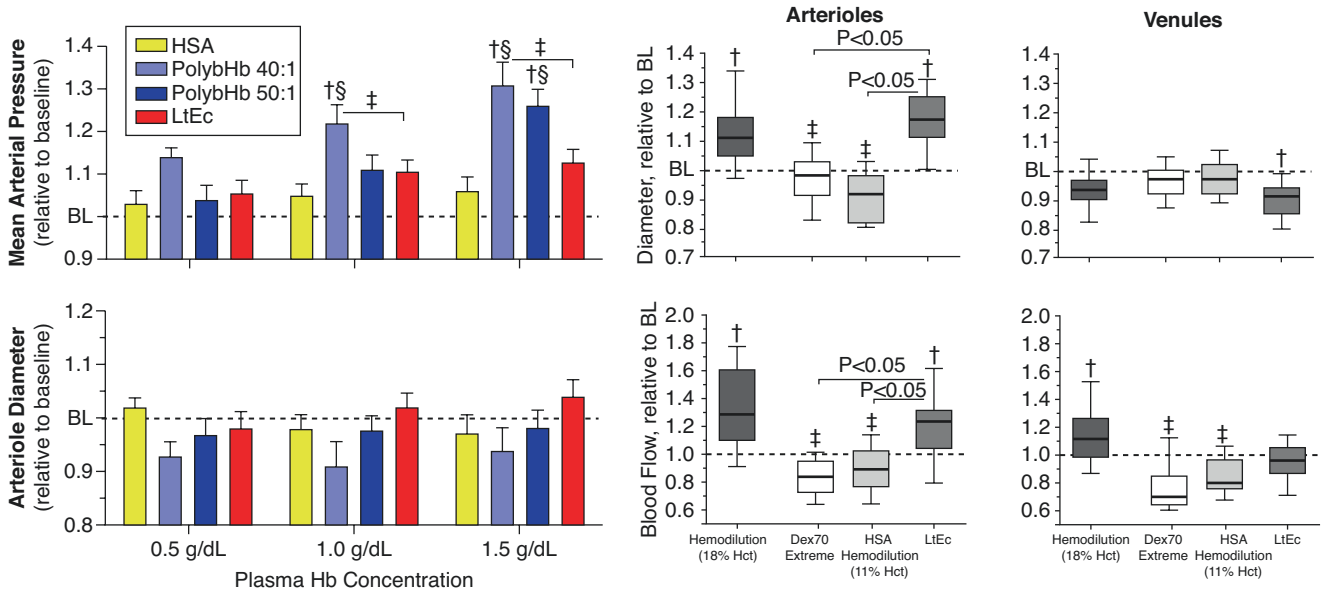


Fig. 26.4 Effects of LtEc, PolybHbs, human serum albumin (HSA), and dextran (Dex70) on mean arterial pressure, blood vessel diameter, and blood flow in topload studies (left) and exchange transfusions (middle/right) in hamsters

vivo, including vasoconstriction, hypertension, and enhanced platelet aggregation [45]. These issues are normally prevented *in vivo* by the RBC membrane, which partitions HbA from the NO in the plasma, but no such protection exists when acellular HBOCs are transfused into the bloodstream.

Since earthworms lack RBCs, LtEc has been forced to adapt to prevent interactions between heme-bound O_2 and other ligands like NO. First of all, it is worth noting that deoxygenated LtEc binds NO, but at a tenfold slower rate than deoxygenated HbA [45]. However, unlike HbA, oxygenated LtEc does not oxidize in the presence of NO *in vitro* [42]. Toploads of LtEc (e.g., 1.5 g LtEc/dL blood) into hamsters also did not appear to induce vasoconstriction or hypertension *in vivo* (Fig. 26.4), while transfusions of polymerized bovine hemoglobins (PolybHbs) significantly increased mean arterial pressure [42]. In contrast, exchange transfusions of LtEc into hamsters induced a slight but significant increase in blood vessel diameter relative to a plasma expander control (Dex70) [43].

Altogether, these results suggest that LtEc can avoid NO dioxygenation while still maintaining an ability to bind O_2 or NO alone. One potential explanation for this unique ability is the molecular structure and small size of the heme pocket found in LtEc [10]. Indeed, the same bulky aromatic acids (Trp or Phe) at the B10 position that resist oxidation of the A, B, and D globins may also stabilize the bound oxygen to protect it from reactions with NO [47] . The heme pockets of each LtEc globin are also more tightly packed with residues than other hemoglobins [10], which may sterically prevent NO and O_2 from occupying the heme pocket at the same time. Both of these unique characteristics have also been observed in acellular hemoglobins from other organisms (e.g., *Lucina pectinata*, [56, 57]) that have lower or negligible rates of reaction with NO. This steric hindrance to NO scavenging could motivate an important paradigm shift from mammalian Hbs to Ecs as RBC substitutes because they avoid the hypertensive effects observed with other HBOCs.

Synthetically Modified LtEc

Although LtEc possesses many natural qualities that would make it an ideal RBC substitute, it might be possible to optimize its properties with synthetic modifications. For example, cross-linking LtEc may further increase its thermal stability, thereby prolonging its potential shelf-life and relaxing its storage requirements. Indeed, cross-linking LtEc with poly(acrylic acid) or glutaraldehyde (GA) significantly increases its thermal stability and protects it from alkaline environments [58, 59].

GA crosslinking has also been used to synthesize several polymerized HBOCs that have been used in clinical trials [60]. Crosslinking with GA has been shown to increase the oxygen affinity, decrease the redox potential, and increase the autoxidation kinetics of HbA [61, 62]. As a result, most studies have involved GA crosslinking of bovine Hb, ovine Hb, and human Hb [63–67]. Although there have been mixed results in regard to animal models, GA crosslinked Hbs have been shown to be more stable than their respective non-crosslinked Hbs [63–65, 67]. For more information on these crosslinked Hbs, please refer to Sect. 4.

In addition to cross-linking, LtEc has also been conjugated to polyethylene glycol (PEG). Transfusions of PEGylated LtEc into hamsters have demonstrated a significant increase in circulation half-life from 18 ± 0.8 hours for native LtEc to 66 ± 1.8 hours for PEGylated LtEc [68]. Furthermore, PEGylation also increased O_2 delivery and blood flow relative to native LtEc without any noticeable side effects [68].

Summary

Overall, LtEc appears to be an attractive RBC substitute that has been shown to safely deliver O_2 in mice and hamsters. It has many unique benefits, including a naturally high MW and stable structure that allows it to resist dissociation, extravasation, and clearance from the bloodstream. The unique structure of its heme pocket also prevents oxidative stress and NO scavenging. Manufacturing of LtEc is also relatively simple, since Canadian nightcrawlers are commercially available in large amounts and high yields of LtEc can be rapidly purified with scalable techniques like tangential flow filtration (TFF) [9, 43, 68]. Nonetheless, additional experiments in higher mammals (e.g., pigs) and human clinical trials must be completed to conclusively determine the efficacy and safety of LtEc as a potential RBC substitute.

Key Points

- Size: The MW of LtEc is high enough to simplify purification and prevent extravasation (but PEG increases circulation half-life further).
- Oxidation: LtEc does not appear to oxidize *in vivo* and can be easily reduced by ascorbic acid.
- NO scavenging: LtEc has a small heme pocket that prevents reactions between O_2 and NO.
- Storage: Deoxygenated LtEc can be stored at 37 °C for up to 7 days without any loss of function.
- Preclinical trials in mice and hamsters have been promising, but LtEc has not yet been tested in higher animals or humans.

References

1. Royer WE, Strand K, van Heel M, Hendrickson WA. Structural hierarchy in erythrocruorin, the giant respiratory assemblage of annelids. Proc Natl Acad Sci U S A. 2000;97(13):7107–11.
2. Mozzarelli A, Bruno S, Ronda L. Biochemistry of hemoglobin. In: Kim HW, Greenburg AG, editors. Hemoglobin-based oxygen carriers as red cell substitutes and oxygen therapeutics. Berlin, Heidelberg: Springer Berlin Heidelberg; 2013. p. 55–73.
3. Storz JF. Evolution of the vertebrate globin gene family. In: Hemoglobin: insights into protein structure, function, and evolution. 1st ed. Oxford: Oxford University Press; 2018. p. 94–123.
4. Svedberg T. Sedimentation constants, molecular weights, and isoelectric points of the respiratory proteins. Cold Spring Harb Symp Quant Biol. 1933;1:51–9.
5. Garlick RL. Structure of annelid high molecular weight hemoglobins (erythrocruorins). Am Zool. 1980;20(1):69–77.
6. Hünefeld FL. Chemical properties in the animal organization. Leipzig: Brockhaus; 1840. p. 160–1.
7. Giegé R. A historical perspective on protein crystallization from 1840 to the present day. FEBS J. 2013;280(24):6456–97.
8. Royer WE, Sharma H, Strand K, Knapp JE, Bhyravbhatla B. Lumbricus erythrocruorin at 3.5 Å resolution: architecture of a megadalton respiratory complex. Structure. 2006;14(7):1167–77.

9. Elmer J, Palmer AF. Biophysical properties of lumbricus terrestris erythrocruorin and its potential use as a red blood cell substitute. J Funct Biomater. 2012;3(1):49–60.
10. Strand K, Knapp JE, Bhyravbhatla B, Royer WE. Crystal structure of the hemoglobin dodecamer from Lumbricus erythrocruorin: allosteric core of giant annelid respiratory complexes. J Mol Biol. 2004;344(1):119–34.
11. Martin PD, Kuchumov AR, Green BN, Oliver RWA, Braswell EH, Wall JS, et al. Mass spectrometric composition and molecular mass of Lumbricus terrestris hemoglobin: a refined model of its quaternary structure. J Mol Biol. 1996;255(1):154–69.
12. Xie Q, Donahue RA, Schneider K, Mirza UA, Haller I, Chait BT, et al. Structure of chain d of the gigantic hemoglobin of the earthworm. Biochim Biophys Acta – Protein Struct Mol Enzymol. 1997;1337(2):241–7.
13. Vinogradov SN. The stoichiometry of the four linker subunits of Lumbricus terrestris hemoglobin suggests an asymmetric distribution. Micron. 2004;35(1–2):127–9.
14. Rose AS, Bradley AR, Valasatava Y, Duarte JM, Prlic A, Rose PW. NGL viewer: web-based molecular graphics for large complexes. Bioinformatics. 2018;34(21):3755–8.
15. Mouche F, Boisset N, Penczek P. a. Lumbricus terrestris hemoglobin – the architecture of linker chains and structural variation of the central toroid. J Struct Biol. 2001;133(2–3):176–92.
16. Fushitani K, Imai K, Riggs AF. Oxygenation properties of hemoglobin from the earthworm, Lumbricus terrestris. Effects of pH, salts, and temperature. J Biol Chem. 1986;261(18):8414–23.
17. Riggs AF, Riggs CK. The self-association of the giant hemoglobin from the earthworm, Lumbricus terrestris. Biochim Biophys Acta – Proteins Proteomics. 2014;1844(6):1071–5.
18. Xu Y, Zheng Y, Fan J-S, Yang D. A new strategy for structure determination of large proteins in solution without deuteration. Nat Methods. 2006;3(11):931–7.
19. Jouan L, Taveau JC, Marco S, Lallier FH, Lamy JN. Occurrence of two architectural types of hexagonal bilayer hemoglobin in annelids: comparison of 3D reconstruction volumes of Arenicola marina and Lumbricus terrestris hemoglobins. J Mol Biol. 2001;305(4):757–71.
20. Royer WE, Omartian MN, Knapp JE. Low resolution crystal structure of arenicola erythrocruorin: influence of coiled coils on the architecture of a megadalton respiratory protein. J Mol Biol. 2007;365(1):226–36.
21. Standley PR, Mainwaring MG, Gotoh T, Vinogradov SN. The calcium, copper and zinc content of some annelid extracellular haemoglobins. Biochem J. 1988;249(3):915–6.
22. Liochev SI, Kuchumov AR, Vinogradov SN, Fridovich I. Superoxide dismutase activity in the giant hemoglobin of the earthworm, Lumbricus terrestris. Arch Biochem Biophys. 1996;330(2):281–4.
23. Kuchumov AR, Loo JA, Vinogradov SN. Subunit distribution of calcium-binding sites in Lumbricus terrestris hemoglobin. J Protein Chem. 2000;19(2):139–49.
24. Kao W-Y, Qin J, Fushitani K, Smith SS, Gorr TA, Riggs CK, et al. Linker chains of the gigantic hemoglobin of the earthworm Lumbricus terrestris: primary structures of linkers L2, L3, and L4 and analysis of the connectivity of the disulfide bonds in linker L1. Proteins Struct Funct Genet. 2006;63(1):174–87.
25. Sharma PK, Kuchumov AR, Chottard GG, Martin PD, Wall JS, Vinogradov SN. The role of the dodecamer subunit in the dissociation and reassembly of the hexagonal bilayer structure of Lumbricus terrestris hemoglobin. J Biol Chem. 1996;271(15):8754–62.
26. Muzzelo C, Neely C, Shah P, Abdulmalik O, Elmer J. Prolonging the shelf life of Lumbricus terrestris erythrocruorin for use as a novel blood substitute. Artif Cells, Nanomed Biotechnol. 2017;46: 39–46.
27. Vinogradov SN, Lugo SD, Mainwaring MG, Kapp OH, Crewe AV. Bracelet protein: a quaternary structure proposed for the giant extracellular hemoglobin of Lumbricus terrestris. Proc Natl Acad Sci U S A. 1986;83(21):8034–8.
28. Matsubara K, Yamaki M, Nagayanma K, Imai K, Ishii H, Gotoh T, et al. Wheat germ agglutinin-reactive chains of giant hemoglobin from the polychaete Perinereis aibuhitensis. Biochim Biophys Acta. 1996;1290:215–23.
29. Ebina S, Matsubara K, Nagayama K, Yamaki M, Gotoh T. Carbohydrate gluing, an architectural mechanism in the supramolecular structure of an annelid giant hemoglobin. Proc Natl Acad Sci U S A. 1995;92(16):7367–71.
30. Zal F, Lallier F, Wall JS, Vinogradov SN, Toulmond A. The multihemoglobin system of the hydrothermal vent tube worm Riftia pachyptila: I. Reexamination of the number and masses of its constituents. J Biol Chem. 1996;271(15):8869–74.
31. Zal F, Küster B, Green BN, Harvey DJ, Lallier FH. Partially glucose-capped oligosaccharides are found on the hemoglobins of the deep-sea tube worm Riftia pachyptila. Glycobiology. 1998;8(7):663–73.
32. Numoto N, Nakagawa T, Ohara R, Hasegawa T, Kita A, Yoshida T, et al. The structure of a deoxygenated 400 kDa haemoglobin reveals ternary- and quaternary-structural changes of giant haemoglobins. Acta Crystallogr Sect D Biol Crystallogr. 2014;70(7):1823–31.
33. Madeira F, Park YM, Lee J, Buso N, Gur T, Madhusoodanan N, et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 2019;47(W1):W636–41.
34. Fushitani K, Matsuura MS, Riggs AF. The amino acid sequences of chains a, b, and c that form the trimer subunit of the extracellular hemoglobin from Lumbricus terrestris. J Biol Chem. 1988;263(14):6502–17.
35. Jhiang SM, Riggs AF. The structure of the gene encoding chain c of the hemoglobin of the earthworm, Lumbricus terrestris. J Biol Chem. 1989;264(32):19003–8.
36. Suzuki T, Riggs AF. Linker chain L1 of earthworm hemoglobin. Structure of gene and protein: homology with low density lipoprotein receptor. J Biol Chem. 1993;268(18):13548–55.
37. Bunn HF, Briehl RW. The interaction of 2,3-diphosphoglycerate with various human hemoglobins. J Clin Invest. 1970;49(6):1088–95.
38. Hirsch RE, Jelicks LA, Wittenberg BA, Kaul DK, Shear HL, Harrington JP. A first evaluation of the natural high molecular weight polymeric Lumbricus terrestris hemoglobin as an oxygen carrier. Artif Cells Blood Substit Immobil Biotechnol. 1997;25(5):429–44.
39. Ochiai T, Weber RE. Effects of magnesium and calcium on the oxygenation reaction of erythrocruorin from the marine polychaete Arenicola marina and the terrestrial oligochaete Lumbricus terrestris. Zool Sci. 2002;19(9):995–1000.
40. Fushitani K, Riggs AF. The extracellular hemoglobin of the earthworm, Lumbricus terrestris: oxygenation properties of isolated chains, trimer, and a reassociated product. J Biol Chem. 1991;266(16):10275–81.
41. Krebs A, Kuchumov AR, Sharma PK, Braswell EH, Zipper P, Weber RE, et al. Molecular shape, dissociation, and oxygen binding of the dodecamer subunit of Lumbricus terrestris hemoglobin. J Biol Chem. 1996;271(31):18695–704.
42. Elmer J, Zorc K, Rameez S, Zhou Y, Cabrales P, Palmer AF. Hypervolemic infusion of Lumbricus terrestris erythrocruorin purified by tangential-flow filtration. Transfusion. 2012;52(8):1729–40.
43. Elmer J, Palmer AF, Cabrales P. Oxygen delivery during extreme anemia with ultra-pure earthworm hemoglobin. Life Sci. 2012;91(17–18):852–9.
44. Savla C, Munoz C, Hickey R, Belicak M, Gilbert C, Cabrales P, et al. Purification of lumbricus terrestris mega-hemoglobin for diverse oxygen therapeutic applications. ACS Biomater Sci Eng. 2020;6(9):4957–68.

45. Roche CJ, Talwar A, Palmer AF, Cabrales P, Gerfen G, Friedman JM. Evaluating the capacity to generate and preserve nitric oxide bioactivity in highly purified earthworm erythrocruorin: a giant polymeric hemoglobin with potential blood substitute properties. J Biol Chem. 2015;290(1):99–117.
46. Zhu H, Ownby DW, Riggs CK, Nolasco NJ, Stoops JK, Riggs AF. Assembly of the gigantic hemoglobin of the earthworm Lumbricus terrestris. Roles of subunit equilibria, non-globin linker chains, and valence of the heme iron. J Biol Chem. 1996;271(47):30007–21.
47. Viana E, Da Silva CHTP, Tabak M, Imasato H, Garratt R. A molecular model for the d chain of the giant haemoglobin from Lumbricus terrestris and its implications for subunit assembly. Biochim Biophys Acta – Protein Struct Mol Enzymol. 1998;1383(1):130–42.
48. Carver T, Brantley R Jr, Singleton E, Arduini R, Quillin M Jr, Phillips G, et al. A novel site-directed mutant of myoglobin with an unusually high O2 affinity and low autooxidation rate. J Biol Chem. 1992;267(20):14443–144450.
49. Dou Y, Maillett DH, Eich RF, Olson JS. Myoglobin as a model system for designing heme protein based blood substitutes. Biophys Chem. 2002;98(1–2):127–48.
50. Jeong ST, Ho NT, Hendrich MP, Ho C. Recombinant hemoglobin(α29leucine → phenylalanine, α96valine → tryptophan, β108sparagine → lysine) exhibits low oxygen affinity and high cooperativity combined with resistance to autoxidation. Biochemistry. 1999;38(40):13433–42.
51. Harrington JP, Kobayashi S, Dorman SC, Zito SL, Hirsch RE. Acellular invertebrate hemoglobins as model therapeutic oxygen carriers: unique redox potentials. Artif Cells Blood Substit Immobil Biotechnol. 2007;35(1):53–67.
52. Dorman SC, Harrington JP, Martin MS, Johnson TV. Determination of the formal reduction potential of Lumbricus terrestris hemoglobin using thin layer spectroelectrochemistry. J Inorg Biochem. 2004;98(1):185–8.
53. Buehler PW, Alayash AI. Oxidation of hemoglobin: mechanisms of control in vitro and in vivo. Transfus Altern Transfus Med. 2007;9(4):204–12.
54. Zimmerman D, DiIusto M, Dienes J, Abdulmalik O, Elmer JJ. Direct comparison of oligochaete erythrocruorins as potential blood substitutes. Bioeng Transl Med. 2017;2(2):212–21.
55. Muzzelo C, Neely C, Shah P, Abdulmalik O, Elmer J. Prolonging the shelf life of Lumbricus terrestris erythrocruorin for use as a novel blood substitute. Artif Cells, Nanomed Biotechnol. 2018;46(1):39–46.
56. Bonaventura C, Henkens R, De Jesus-Bonilla W, Lopez-Garriga J, Jia Y, Alayash AI, et al. Extreme differences between Hemoglobins I and II of the Clam Lucina pectinalis in their reactions with nitrite. Biochim Biophys Acta – Proteins Proteomics. 2010;1804(10):1988–95.
57. De Jesús-Bonilla W, Jia Y, Alayash AI, López-Garriga J. The heme pocket geometry of Lucina pectinata hemoglobin II restricts nitric oxide and peroxide entry: model of ligand control for the design of a stable oxygen carrier. Biochemistry. 2007;46(37):10451–60.
58. Spivack K, Tucker M, Zimmerman D, Nicholas M, Abdulmalik O, Comolli N, et al. Increasing the stability of Lumbricus terrestris erythrocruorin via poly(acrylic acid) conjugation. Artif Cells, Nanomed Biotechnol. 2018;46(sup2):1137–44.
59. Rajesh A, Zimmerman D, Spivack K, Abdulmalik O, Elmer J. Glutaraldehyde cross-linking increases the stability of Lumbricus terrestris erythrocruorin. Biotechnol Prog. 2018;34(2):521–8.
60. Jahr JS, Walker V, Manoochehri K. Blood substitutes as pharmacotherapies in clinical practice. Curr Opin Anaesthesiol. 2007;20(4):325–30.
61. Guillochon D, Vijayalakshmi MW, Thiam-Sow A, Thomas D. Effect of glutaraldehyde on hemoglobin: functional aspects and Mossbauer parameters. Biochem Cell Biol. 1986;64(1):29–37.
62. Chevalier A, Guillochon D, Nedjar N, Piot JM, Vijayalakshmi MW, Thomas D. Glutaraldehyde effect on hemoglobin: evidence for an ion environment modification based on electron paramagnetic resonance and mossbauer spectroscopies. Biochem Cell Biol. 1990;68(4):813–8.
63. Buehler PW, Boykins RA, Jia Y, Norris S, Freedberg DI, Alayash AI. Structural and functional characterization of glutaraldehyde-polymerized bovine hemoglobin and its isolated fractions. Anal Chem. 2005;77(11):3466–78.
64. Zhang N, Jia Y, Chen G, Cabrales P, Palmer AF. Biophysical properties and oxygenation potential of high-molecular-weight glutaraldehyde-polymerized human hemoglobins maintained in the tense and relaxed quaternary states. Tissue Eng – Part A. 2011;17(7–8):927–40.
65. Gotshall RW, Hamilton KL, Foreman B, Van Patot MCT, Irwin DC. Glutaraldehyde-polymerized bovine hemoglobin and phosphodiesterase-5 inhibition. Crit Care Med. 2009;37(6):1988–93.
66. Buehler PW, Alayash AI. All hemoglobin-based oxygen carriers are not created equally. Biochim Biophys Acta – Proteins Proteomics. 2008;1784(10):1378–81.
67. Farcas AD, Toma VA, Roman I, Sevastre B, Scurtu F, Silaghi-Dumitrescu R. Glutaraldehyde-polymerized hemoglobin: in search of improved performance as oxygen carrier in hemorrhage models. Bioinorg Chem Appl. 2020;1–11.
68. Jani VP, Jelvani A, Moges S, Nacharaju P, Roche C, Dantsker D, et al. Polyethylene glycol camouflaged earthworm hemoglobin. PLoS One. 2017;12(1):1–22.

Part IV

Products, Not Approved, in Progress, and Approved for Human/Veterinary Use

HemAssist: Development, Clinical Trials, Lessons Learned

27

Timothy N. Estep

Introduction

HemAssist® development was initiated under the auspices of a contract between the United States Army Medical Research and Development Command (USAMRDC) and Baxter International Corporation from 1985 to 1988 [1]. This effort entailed a close collaboration between researchers at the Letterman Army Institute of Research (LAIR) and Baxter. During the course of this effort a mutual decision was made to focus on the production of a specific HBOC discovered at the University of Iowa [2]. The active principle was human hemoglobin crosslinked between the two alpha subunits by a fumarate crosslinker derived from the diaspirin reagent, bis (3,5 dibromosalicyl) fumarate (DBBF). Hence, this product was initially denoted as diaspirin crosslinked hemoglobin (DCLHb). This particular derivative was chosen because a high yield was obtained of a specific hemoglobin derivative which was stabilized to enhance intravascular persistence and exhibited oxygen binding and release characteristics which closely mimicked those of fresh whole blood [2, 3].

Production scale up of DCLHb entailed the identification and implementation of purification procedures which could be performed at large scale, development of an improved method for the synthesis of large quantities of highly purified DBBF, development and validation of both in-process and final release assays, and the integration and validation of virus removal and inactivation steps [4]. The resulting process consistently produced DCLHb from human red cells with an overall yield in excess of 50%, and which was sterile, highly crosslinked, and exhibited low levels of non-hemoglobin proteins and process residuals [4]. As a consequence of these efforts, over 200 liters of 10 g/dl DCLHb solution was delivered to LAIR [1]. This material was used in a variety of preclinical assessments at LAIR [1, 5, 6] and laboratories receiving material from LAIR [7–9]. Ultimately, commercial scale production of DCLHb was demonstrated in a purpose built facility in Neuchatel, Switzerland, and validation studies demonstrated a total level of virus reduction of at least 100 billion fold for an array of virus challenges [10, 11].

In addition to the experiments performed at LAIR, a number of studies were performed at Baxter, as well as academic and contract laboratories collaborating with Baxter. Results were sufficiently encouraging that Baxter senior management decided to invest considerable company resources into the further development of DCLHb after the conclusion of the contract with USAMRDC. The US ARMY also decided to pursue further assessment of DCLHb using material produced at LAIR and, subsequently, the Walter Reed Army Institute of Research [12, 13]. The production of DCLHb by the ARMY was initially based on the complete set of standard operating procedures which was conveyed to the ARMY as part of the Baxter/USAMRDC contract. However, both the ARMY and Baxter made subsequent changes to their respective manufacturing processes so that these diverged significantly [4, 12, 13]. On the basis of published data, the Baxter product was more highly crosslinked and contained lower levels of contaminating pyrogens and phospholipids (Table 27.1). The ARMY also changed the composition of their final formulation, replacing lactate with acetate [12, 13]. Acetate has pharmacologic activities which have led to discontinuation of its use in dialysis due to myocardial depression, hypotension, hyopnea, and hypoxemia [14].

Table 27.1 Comparison of DCLHb (HemAssist) produced by Baxter and ααHb produced by the US Army

Parameter (Units)	Army[a]	Baxter[b]
Total hemoglobin (g/dL)	9.8–9.95	10.2
Methemoglobin (%)	3.2–7.5	3.2
Desired product (%)[c]	50–90+	99.8
Endotoxin (EU/mL)	0.1–0.2	<0.06
Rabbit pyrogen test (% pass)	63–100	100
Phospholipid (ppm)	0.75–1.0	0.1

[a]Data taken from references [12, 13]. [b]Data taken from reference [4]. [c]Defined as the amount of desired alpha-alpha crosslinked hemoglobin

T. N. Estep (✉)
Chart Biotech Consulting, LLC, Erie, CO, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_27

Thus, it is not surprising that these two, ostensibly similar, HBOC formulations exhibit different physiologic properties, the most striking of which is the fact that the Baxter product causes significant blood volume expansion after infusion [15], whereas the ARMY product causes blood volume contraction [16, 17]. Unfortunately, the distribution of two different diaspirin crosslinked formulations amongst researchers has caused confusion in the field, especially since these have sometimes been identified by the same nomenclature (e.g. αα crosslinked hemoglobin). All subsequent observations discussed in this chapter are based on results obtained with the Baxter manufactured product which is denoted interchangeably as DCLHb or HemAssist.

Preclinical Testing

The availability of large quantities of high quality product enabled extensive safety and efficacy testing. Efficacy was demonstrated in animal models of high volume blood replacement [18], resuscitation from hemorrhagic shock [19], resuscitation from cardiac arrest [20], treatment of cerebral [21] and spinal cord [22] ischemia, and support of cardiac function during balloon angioplasty [23]. DCLHb was also well tolerated during extensive whole animal and organ specific toxicity testing. Transient increases in some enzymes were observed, but these resolved rapidly. Centrolobular necrosis was detected in the liver, but this also resolved rapidly. Notably, DCLHb had little adverse effect on kidney pathology or function [3]. However, two other issues were identified during the preclinical testing of DCLHb which prompted further investigations.

The first of these was the fact that DCLHb was vasoactive, resulting in elevations of blood pressure in excess of those explicable on the basis of blood volume replacement or expansion [24]. Subsequent characterization of this phenomenon demonstrated that consumption of nitric oxide (NO) by extravasated hemoglobin is the primary mechanism for this hypertension [25]. Physiologic measurements using radioactive microspheres showed that vasoconstriction after DCLHb infusion varies significantly between organs and tissues, being pronounced in skeletal muscle, less in many other tissues, and, importantly, nonexistent in heart vessels [26]. The pressor response to DCLHb is manifested at low infused concentrations and rises to a maximum of approximately 40% of baseline at intermediate doses, with no further increase at higher DCLHb concentrations [24]. This increase could be mitigated by commonly used antihypertensive agents. Ultimately, the regulatory position on this issue was that patient risk was sufficiently low that clinical trials could proceed, albeit with close monitoring of hemodynamics.

A second finding of concern was the appearance of microscopic cardiac lesions in certain species [27]. This finding prompted an extensive testing program to characterize pathology, explore potential mechanisms, and define consequences. These studies ruled out coronary vasoconstriction and infarction as mechanisms, and demonstrated that the lesion resulted in no detectable adverse effect on cardiac electrophysiology or function, even in the most sensitive species. In light of these results the risk of significant harm to humans was deemed acceptable and clinical testing was initiated. During this time period DCLHb was trade named "HemAssist™".

Clinical Testing

Low doses of HemAssist infused into normal volunteers were well tolerated, with modest, but expected, increases in blood pressure and no evidence of adverse cardiac events [28]. On the basis of these results, as well as those of preclinical evaluations, it was decided to focus subsequent clinical trials on the indications of blood replacement and resuscitation from hemorrhagic shock.

HemAssist was evaluated as a replacement for blood transfusion in both cardiac [29] and noncardiac surgery patients [30, 31]. In cardiac surgery patients, the overall number of packed erythrocyte units (pRBCs) transfused did not differ between treated and control groups, but 19% of treated patients did avoid any pRBC infusion (versus 0% of controls) when up to 750 mL of HemAssist was utilized [29]. Treated patients experienced modest increases in mean arterial and pulmonary artery pressures and a decrease in cardiac output, but these were not associated with clinical sequelae. Jaundice/hyperbilirubinemia, hematuria, and increased liver enzymes were expected observations in treated patients as a consequence of increased hemoglobin metabolism. There was no elevation in cardiac damage specific enzymes relative to controls, but some increase in lipase and pancreatic-specific amylase. No cases of pancreatitis were observed in this study.

In a small trial with noncardiac surgery patients, there was no evidence of cardiac ischemia, cardiac infarction, stroke, or pulmonary edema, although blood pressure was elevated for 24–30 hours after DCLHb infusion [30]. More (7/12) DCLHb treated patients were treated with antihypertensives than controls (2/12). Jaundice, elevated bilirubin levels, and asymptomatic hematuria were again observed more frequently in treated patients, as were transient elevations in serum lactate dehydrogenase, aspartate transaminase, creatine kinase, and amylase. Renal function appeared to be well maintained, but three treated patients experienced ileus and one was diagnosed with mild pancreatitis.

The most rigorous assessment of DCLHb in the noncardiac surgical setting was a randomized, prospective, double-blinded Phase III trial by Schubert and coworkers [31].

Utilization of up to 750 mL of DCLHb solution in lieu of blood transfusion resulted in complete avoidance of the latter in 23% of treated patients after seven days. A statistically significant ($p = 0.002$) reduction in overall pRBC transfusion was also observed, with an average of two units required in treated patients versus three in controls. Out of the 181 patients enrolled at 19 clinical sites, mortality (4% versus 3%) and the incidence of adverse events (21% versus 15%) were similar between DCLHb treated patients and controls, respectively. However, the incidence of jaundice, urinary side effects, and pancreatitis was more frequent in patients infused with HemAssist. This study was terminated early due to safety concerns about pancreatitis and a mortality imbalance observed during initial patient enrollment in a contemporaneous study of DCLHb in trauma victims.

With respect to the latter, HemAssist was evaluated in three clinical trials in the treatment of hemorrhagic shock. In a Phase II safety study, patients with Class II-IV hemorrhagic in hypovolemic shock within were infused with 50, 100 or 200 mL of a 10% DCLHb solution or normal saline, followed by standard of care [32]. Lower mortality was observed in the DCLHb versus control patients (13/71 versus 16/68), but the difference was not statistically significant. The incidence of adverse events and serious adverse events was similar between groups. There was no evidence of renal insufficiency or cardiac ischemia in treated patients. At the 200 mL dose there were greater increases in serum amylase, lactate dehydrogenase, and creatine kinase myocardial subfraction levels in treated patients, but these were not considered to be clinically significant. On the basis of these results, two Phase III studies were initiated in the US and Europe [33, 34]. Although the same formulation was used in both studies, there were differences in the clinical protocols and clinical outcomes. The US study was halted after the infusion of only 98 patients total, out of a planned 850, due to the fact that 24 treated patients had died, compared to 8 in the control arm of the study. This outcome ultimately resulted in the decision to terminate all DCLHb development. However, a subsequent analysis of patient deaths in the US trial published 3 years later by the lead study investigators suggested that all but two of the 32 patient deaths would be expected on the basis of the type and severity of patient injuries, one in each group [35]. Interestingly, a substantial fraction of the mortality imbalance occurred in the initial patients treated at the 16 study sites that eventually enrolled at least one patient [36]. Also notable is the fact that mortality in the European trial after a comparable level of enrollment (121 patients) was not significantly different between treatment and control groups (42% and 38%, respectively) [34]. Nevertheless, the European trial was terminated as a consequence of the US trial experience. Further analysis of the US trial data suggested that vasoactivity was not an important contributor to the mortality imbalance as the average blood pressure immediately after resuscitation was quite similar between treated and control patients [37]. Moreover, DCLHb infusion did not result in higher lactate or base deficit levels, implying no adverse effects on organ perfusion [38].

Although the small number of patients enrolled in the Phase III resuscitation trials does not permit definitive conclusions, comparison of differences between the US and European protocols (Table 27.2) suggest several interesting hypotheses [39]. One difference is that the US trial protocol permitted the enrollment of patients who had suffered cardiac arrest, while such patients were excluded from the European study. Experience has shown that such patients have a greater than 90% chance of mortality [40]. As it happened, 12 such patients were enrolled in the US study, but the majority of these (10 versus 2) were randomized to the treatment arm [35]. Another potentially important difference was that treatment of patients was begun on-scene in the European study, but only after admission to the emergency room in the US. A third difference was that European patients were excluded from enrollment if they had received one liter or more of other fluids, while no such restriction was placed on US patients. As a consequence, US patients may have received substantially more total fluid volume than their European counterparts [39]. This is particularly noteworthy in light of subsequent results obtained in sheep resuscitation protocols which showed that HemAssist was a much more potent volume expander than a comparable volume of oncotically matched albumin solution [41]. Thus, the combination of an unexpectedly large HemAssist volume expansion effect coupled with larger infused volumes of additional fluids could have resulted in over resuscitation of some treated patients in the US trial. Yet another potentially important difference is the fact that US patients suffered more penetrating injuries. As noted by Kerner et al., such patients may be particularly susceptible to aggressive fluid resuscitation [34]. Finally, it is notable that the mortality in both treated groups and the European control group were similar to the targeted patient mortality risk of 40%, but the mortality in the US control group was significantly

Table 27.2 Comparison of protocols and outcomes in Phase III trials of HemAssist (DCLHb) in the US and Europe[a]

Study	Europe	US
Site of treatment	On-scene	Trauma center
Volume DCLHb infused (mL)	250–1000	500–1000
Limitation on previous fluid volume Infused	Yes	No
Previous cardiac arrest as exclusion Criteria	Yes	No
28-day mortality:		
Treated (deaths/n)(%)	22/52 (42%)	24/52 (46%)
Controls (deaths/n)(5)	22/58 (38%)	8/46 (17%)

[a]Data taken from references [33, 34]

lower, suggesting some confounding factors were present in the US trial [33]. A more comprehensive discussion of HemAssist clinical development has been presented by Przybelski [42].

Subsequent Analyses – Myocardial Infarction

Although HemAssist development was terminated in 1999, data from clinical trials were included in a meta-analysis of HBOC toxicity [43]. This analysis suggested that HBOCs as a class increase the risk of mortality and myocardial infarction (MI) in treated patients. With respect to HemAssist, the mortality imbalance derives almost entirely from the US Phase III trauma study, which, as noted above, is significantly confounded. Thus, the mortality risk associated with the use of this product is unclear.

More suggestive conclusions may be drawn with respect to myocardial infarction, assuming that the reported incidence of MI in the HBOC clinical literature is positively correlated with the actual occurrence of MI [44]. This qualifier is prompted by the fact that hemoglobin and hemoglobin metabolites may interfere with the analysis of troponin, a primary marker of MI in contemporary cardiology. With this reservation, additional comprehensive analysis of clinical trial results implies that HBOCs as a class probably do increase the risk of MI in certain patients because this risk is positively correlated with both HBOC dose and molecular weight [44]. Consideration of possible mechanisms suggests that vasoconstriction is unlikely, since HBOCs do not reduce coronary blood flow [44]. MI risk is also probably unrelated to the generation of microscopic heart lesions, because these lesions were never found to be associated with areas of infarct [27]. Furthermore, the molecular weight dependency of MI risk between different HBOCs is exactly opposite that for microscopic lesion development [27, 44]. However, other mechanisms are possible, including the enhancement of intravascular thrombosis risk due to exacerbation of endothelial dysfunction by heme released from oxidized hemoglobin, platelet activation due to the consumption of nitric oxide and/or oxidative stress, and inhibition of the breakdown of ultralarge von Willebrand factor [45]. Intravascular etiology is supported by the positive correlation between HBOC molecular weight and MI incidence, since larger HBOCs are known to persist in the circulation longer [46], and therefore any untoward interactions within the vascular space or with endothelium are more likely to occur. Thus, HemAssist, like other chemically modified HBOCs, is probably associated with increased risk of MI, a risk which was not apparent during earlier analyses of individual clinical trial data.

Summary and Lessons Learned

Highly purified solutions of DCLHb (HemAssist) were efficiently produced at large scale and performed well in standard safety testing in healthy animals. In addition, efficacy was demonstrated in a variety of animal models of stroke and hemorrhagic shock. The safety profile also seemed acceptable in Phase I and II human trials, but several issues were identified in more demanding Phase III studies. This suggests that a more systematic progression into the latter is appropriate, because the learning curve is steep [36], particularly with a totally new class of products. HBOCs are different from both red cells and traditional intravenous solutions and it is unclear whether the protocols used for HemAssist were optimal, despite substantial efforts expended in their design and implementation [34, 35, 41]. Several clinicians have argued that higher doses may have been appropriate in the resuscitation of some patients [34]. Thus, future HBOC clinical trials should be designed with regard to posology, patient selection and concomitant therapies (e.g. intravenous fluids) in light of these results.

With respect to safety, it is remarkable that at doses which far exceed those of other pharmacological agents, HemAssist was generally well tolerated in a variety of animal models and human patients. Nevertheless, HemAssist was not devoid of risk, and it should not be surprising that a new finding would emerge as a larger body of patients was evaluated. The incidence of MI in control patients enrolled in HemAssist clinical trials averaged 1%, while in treated patients it was 1.6%. With other HBOCs the incidence in treated patients was higher [44]. Thus, there is legitimate cause for clinical and regulatory concern with respect to the safety of these products. On the other hand, the fact that most treated patients do not experience MI, there is demonstrable blood sparing in some patient populations [31], and anecdotal evidence that HBOCs can support cardiac function and sustain life in dire situations [47, 48], suggests that this class of therapy merits further development. Aside from the continued development of HBOC derivatives with improved safety characteristics, better understanding of how to best use these products and more refined selection of those patients most likely to benefit from HBOC treatment are warranted.

Key Points

- HemAssist development demonstrated that a highly crosslinked, highly purified human HBOC formulation

with extremely low risk of pathogen transmission could be efficiently produced at large scale.

- Different formulations of diaspirin crosslinked hemoglobin produced in different facilities may elicit different physiologic responses.
- Preclinical testing demonstrated low toxicity when HemAssist was infused into healthy animals from a variety of species and the ability to reverse whole animal and specific organ ischemia in models of hemorrhagic shock, stroke, and organ perfusion.
- In human testing, HemAssist was well tolerated at low doses in normal human volunteers and moderate doses in most patients.
- Blood sparing was demonstrated in some, but not all, patient populations.
- The increase in systemic blood pressure as a consequence of the vasoactivity of HemAssist was generally well tolerated and manageable.
- Several safety concerns arose during later stage clinical testing suggesting that HemAssist infusion may be associated with an increased risk of pancreatitis, myocardial infarction, and mortality in some patients.
- Comparison of the protocols and patient enrollment criteria between US and European Phase III trials of HemAssist in the treatment of hemorrhagic shock suggest that higher treated patient mortality in the former was due to an imbalance with respect to risk between treated and control patients and, possibly, suboptimal fluid management.
- There is a substantial learning curve associated with the optimal use of HBOCs in extremely ill patients.

References

1. Winslow RM. αα-Crosslinked human hemoglobin as a model red cell substitute. In: Sekiguchi S, editor. Red cell substitutes the proceedings of the second international symposium of red cell substitutes. Tokyo: Kindai shuppan; 1991. p. 68–80.
2. Chatterjee R, Welty EV, Walder RY, Pruitt SL, Rogers PH, Arnone A, et al. Isolation and characterization of a new hemoglobin derivative cross-linked between the α chains (lysine 99 $\alpha_1 \rightarrow$ lysine 99 α_2). J Biol Chem. 1986;261(21):9929–37. PMID: 3090027.
3. Nelson DJ. HemAssist™: development and clinical profile. In: Rudolph AS, Rabinovici R, Feuerstein GZ, editors. Red blood cell substitutes basic principles and clinical applications. New York: Marcel Dekker; 1998. p. 353–400.
4. Azari M, Rohn K, Picken J. Diaspirin crosslinked hemoglobin (DCLHb™): characterization of the process and the product manufactured under GMP requirements for clinical studies. Artif Cells Blood Substit Immobil Biotechnol. 1994;22(3):701–8. https://doi.org/10.3109/10731199409117901. PMID: 7994391.
5. Hess JR, Wade CE, Winslow RM. Filtration-assisted exchange transfusion using alpha alpha Hb, an erythrocyte substitute. J Appl Physiol (1985). 1991;70(4):1639–44. https://doi.org/10.1152/jappl.1991.70.4.1639. PMID 1905289.
6. Smith CD, Schuschereba ST, Hess JR, McKinney L, Bunch D, Bowman PD. Liver and kidney injury after administration of hemoglobin cross-linked with bis(3,5-dibromosalicyl) fumarate. Biomater Artif Cells Artif Organs. 1990;18(2):251–61. https://doi.org/10.3109/10731199009117305. PMID: 2369649.
7. Przybelski RJ, Malcolm DS, Burris DG, Winslow RM. Cross-linked hemoglobin solution as a resuscitative fluid after hemorrhage in the rat. J Lab Clin Med. 1991;117(2):143–51. PMID: 1993856.
8. Urbaitis BK, Razynska A, Corteza Q. Fronticelli, Bucci E. intravascular retention and renal handling of purified natural and intramolecularly cross-linked hemoglobins. J Lab Clin Med. 1991;117(2):115–21. PMID: 1993852.
9. Starnes HF, Tewari A, Flokas K, Kosek JC, Brown D, Van-Kessel AL, et al. Effectiveness of a purified human hemoglobin as a blood substitute in the liver. Gastroenterology. 1991;101(5):1345–53. https://doi.org/10.1016/0016-5085(91)90087-2. PMID: 1936806.
10. Azari M, Ebeling A, Baker R, Burhop K, Camacho T, Estep T, et al. Validation of the heat treatment step used in the production of diaspirin crosslinked hemoglobin (DCLHb™) for viral inactivation. Artif Cells Blood Substit Immobil Biotechnol. 1998;26(5–6):577–82. https://doi.org/10.3109/10731199980117477. PMID: 9844723.
11. Azari M, Boose JA, Burhop KE, Camacho T, Catarello J, Darling A, et al. Evaluation and validation of virus removal by ultrafiltration during the production of diaspirin crosslinked haemoglobin (DCLHb). Biologicals. 2000;28(2):81–94. https://doi.org/10.1006/biol.2000.0246. PMID: 10885615.
12. Winslow RM, Chapman KW. Pilot-scale preparation of hemoglobin solutions. In: Everse J, Vandegriff KD, Winslow RM, editors. Methods in enzymology, volume 231 hemoglobin part B biochemical and analytical methods. San Diego: Academic; 1994. p. 3–16.
13. Highsmith FA, Driscoll CM, Chung BC, Chavez MD, Macdonald VW, Manning JM, et al. An improved process for the production of sterile modified haemoglobin solutions. Biologicals. 1997;25(3):257–68. https://doi.org/10.1006/biol.1997.0096. PMID: 9324994.
14. Neavyn MJ, Boyer EW, Bird SB, Babu KM. Sodium acetate as a replacement for sodium bicarbonate in medical toxicology: a review. J Med Toxicol. 2013;9(3):250–4. https://doi.org/10.1007/s13181-013-0304-0. PMID: 23636658.
15. Fischer SR, Burnet M, Traber DL, Prough DS, Kramer GC. Plasma volume expansion with solutions of hemoglobin, albumin, and ringer lactate in sheep. Am J Phys. 1999;276(6):H2194–203. https://doi.org/10.1152/ajpheart.1999.276.6.H2194. PMID: 10362704.
16. Migita R, Gonzales A, Gonzales ML, Vandegriff KD, Winslow RM. Blood volume and cardiac index in rats after exchange transfusion with hemoglobin-based oxygen carriers. J Appl Physiol (1985). 1997;82(6):1995–2002. https://doi.org/10.1152/jappl.1997.82.6.1995. PMID: 9173969.
17. Caron A, Mayer JC, Menu P, Alayash A, Marie PY, Vigneron C. Measurement of blood volume after haemodilution with haemoglobin-based oxygen carriers by a radiolabeled-albumin method. Transfus Med. 2001;11(6):433–42. https://doi.org/10.1046/j.1365-3148.2001.00337.x. PMID: 11851941.
18. Hutter J, Sinitsina I, Hermann J, Messmer K. Total blood exchange with diaspirin cross-linked hemoglobin is compatible with long-term survival. In: Messmer K, Burhop KE, Hutter J, editors. Microciculatory effects of hemoglobin solutions, Prog Appl Microcirc. Basel: Karger, vol. 25; 2004. p. 39–47.
19. Habler O, Kleen M, Pape A, Meisner F, Kemming G, Messmer K. Diaspirin-crosslinked hemoglobin reduces mortality of severe hemorrhagic shock in pigs with critical coronary stenosis. Crit Care Med. 2000;28(6):1889–98. https://doi.org/10.1097/00003246-00034. PMID: 10890638.

20. Chow MS, Fan C, Tran H, Zhao H, Zhou L. Effects of diaspirin cross-linked hemoglobin (DCLHb) during and post-CPR in swine. J Pharmacol Exp Ther. 2001;297(1):224–9. PMID: 11259548.
21. Cole DJ, Schell RM, Drummond JC, Reynolds L. Focal cerebral ischemia in rats. Effect of hypervolemic hemodilution with diaspirin cross-linked hemoglobin versus albumin on brain injury and edema. Anesthesiology. 1993;78(2):335–42.
22. Bowes MP, Burhop KE, Zivin JA. Diaspirin cross-linked hemoglobin improves neurological outcome following reversible but not irreversible CNS ischemia in rabbits. Sroke. 1994;25(11):2253–7. https://doi.org/10.1161/01.str.25.11.2253. PMID 7974553.
23. McKenzie JE, Cost EA, Scandling DM, Ahle NW, Savage RW. Effects of diaspirin crosslinked haemoglobin during coronary angioplasty in the swine. Cardiovasc Res. 1994;28(8):1188–92. https://doi.org/10.1093/cvr/28.8.1188. PMID 7954621.
24. Malcolm DS, Hamilton IN, Schultz SC, Cole F, Burhop K. Characterization of the hemodynamic response to intravenous diaspirin crosslinked hemoglobin solution in rats. Artif Cells Blood Substit Immobil Biotechnol. 1994;22(1):91–107. https://doi.org/10.3109/10731199409117402. PMID: 8055101.
25. Doherty DH, Doyle MP, Curry SR, Vali RJ, Fattor TJ, Olson JS, et al. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. Nat Biotechnol. 1998;16(7):672–6. https://doi.org/10.1038/nbt0798-672. PMID 9661203.
26. Sharma AC, Rebello S, Gulati A. Regional circulatory and systemic hemodynamic effects of diaspirin cross-linked hemoglobin in the rat. Artif Cells Blood Substit Immobil Biotechnol. 1994;22(3):593–602. https://doi.org/10.3109/10731199409117888. PMID: 7994379.
27. Burhop K, Gordon D, Estep T. Review of hemoglobin-induced myocardial lesions. Artif Cells Blood Substit Immobil Biotechnol. 2004;32(3):353–74. https://doi.org/10.1081/bio-200027429. PMID: 15508274.
28. Przybelski RJ, Daily EK, Kisicki JC, Mattia-Goldberg C, Bounds MJ, Colburn WA. Phase I study of the safety and pharmacologic effects of diaspirin cross-linked hemoglobin solution. Crit Care Med. 1996;24(12):1993–2000. https://doi.org/10.1097/00003246-19961200011. PMID: 8968267.
29. Lamy ML, Daily EK, Brichant JF, Larbuisson RP, Demeyere RH, Vandermeersch EA, et al. Randomized trial of diaspirin cross-linked hemoglobin solution as an alternative to blood transfusion after cardiac surgery. Anesthesiology. 2000;92(3):646–56. https://doi.org/10.1097/00000542-200003000-00007. PMID: 1079942.
30. Schubert A, O'Hara JF, Przybelski RJ, Tetzlaff JE, Marks KE, Mascha E, et al. Effect of diaspirin crosslinked hemoglobin (DCLHb HemAssist™) during high blood loss surgery on selected indices of organ function. Artif Cells Blood Substit Immobil Biotechnol. 2002;30(4):259–83. https://doi.org/10.1081/bio-120006118. PMID 12227646.
31. Schubert A, Przybelski RJ, Eidt JF, Lasky LC, Marks KE, Karafa M, et al. Diaspirin-crosslinked hemoglobin reduces blood transfusion in noncardiac surgery: a multicenter, randomized, controlled, double-blinded trial. Anesth Analg. 2003;97(2):323–32. https://doi.org/10.1213/01.ane.0000068888.02977.da. PMID: 12873912.
32. Przybelski RJ, Daily EK, Micheels J, Sloan E, Mols P, Corne L, et al. A safety assessment of diaspirin cross-linked hemoglobin (DCLHb) in the treatment of hemorrhagic, hypovolemic shock. Prehosp Disaster Med. 1999;14(4):251–64. PMID: 10915412.
33. Sloan EP, Koenigsberg M, Gens D, Cipolle M, Runge J, Mallory MN, et al. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock. JAMA. 1999;282(19):1857–64. https://doi.org/10.1001/jama.282.19.1857. PMID: 1053278.
34. Kerner T, Ahlers O, Veit S, Riou B, Saunders M. Pison, et al. DCL-Hb for trauma patients with severe hemorrhagic shock: the European "on-scene" multicenter study. Intensive Care Med. 2003;29(3):378–85. https://doi.org/10.1007/s00134-002-1622-x. PMID 12541156.
35. Sloan EP, Koenigsberg M, Brunett PH, Bynoe RP, Morris JA, Tinkoff G, et al. Post hoc mortality analysis of the efficacy trial of diaspirin cross-linked hemoglobin in the treatment of severe traumatic hemorrhagic shock. J Trauma. 2002;52(5):887–95. https://doi.org/10.1097/00005373-200205000-00011. PMID: 11988654.
36. Sloan EP, Koenigsberg, DCLHb Traumatic Hemorrhagic Shock Study Group, University of Illinois at Chicago. Analysis of the first and subsequently enrolled patients in the DCLHb traumatic hemorrhagic shock clinical trial [abstract]. Acad Emerg Med. 2000;7(5):503a.
37. Sloan EP, Philbin NB, Koenigsberg MD, Gao W, DCLHb Traumatic Hemorrhagic Shock Study Group. European HOST Investigators The lack of consistent diaspirin cross-linked hemoglobin infusion blood pressure effects in the US and EU traumatic hemorrhagic shock clinical results. Shock. 2010:123–33. https://doi.org/10.1097/shk.0b013e3181ac482b. PMID: 20092028.
38. Sloan EP, Koenigsberg MD, Philbin NB, Gao W. DCLHb traumatic hemorrhagic shock study groups; European HOST investigators. Diaspirin cross-linked hemoglobin infusion did not influence base deficit and lactic acid levels in two clinical trials of traumatic hemorrhagic shock patient resuscitation. J Trauma. 2010;68(5):1158–71. https://doi.org/10.1097/TA.0b013e3181bbfaac. PMID: 20145575.
39. Sloan EP. The clinical trials of diaspirin cross-linked hemoglobin (DCLHb) in severe traumatic hemorrhagic: the tale of two continents. Intensive Care Med. 2003;29(3):347–9. https://doi.org/10.1007/s00134-003-1637-y. PMID: 12710459.
40. Evans CCD, Petersen A, Meier EN, Buick JE, Schreiber M, Kannas D, et al. Resuscitation outcomes consortium investigators. Prehospital traumatic cardiac arrest: management and outcomes from the resuscitation outcomes consortium epistry-trauma and PROPHET registries. J Trauma Acute Care Surg. 2016;81(2):285–93. https://doi.org/10.1097/TA.0000000000001070. PMID: 27070438.
41. Fischer SR, Burnet M, Traber DL, Prough DS, Kramer GC. Plasma volume expansion with solutions of hemoglobin, albumin, and ringer lactate in sheep. Am J Phys. 1999;276(6):H2194–203. https://doi.org/10.1152/ajpheart.1999.276.6.H2194. PMID: 10362704.
42. Przybelski RJ. Clinical studies with DCLHb. In: Winslow RM, editor. Blood substitutes. London: Academic; 2006. p. 415–23.
43. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death. JAMA. 2008;299(19):2303–12. https://doi.org/10.1001/jama.299.19.jrv80007. PMID: 18443023.
44. Estep TN. Haemoglobin-based oxygen carriers and myocardial infarction. Artif Cells Nanomed Biotechnol. 2019;47(1):593–601. https://doi.org/10.1080/21691401.2019.1573181. PMID: 30849245.
45. Estep TN. Hemoglobin-based oxygen carriers and myocardial infarction: assessment of potential mechanisms. In: Chang et al., editors. Nanobiotherapeutic based blood substitutes. New Jersey: World Scientific; 2021. p. 279–311. https://doi.org/10.1142/12054.
46. Estep TN. Pharmacokinetics and mechanisms of plasma removal of hemoglobin-based oxygen carriers. Artif Cells Nanomed Biotechnol. 2015;43(3):203–15. https://doi.org/10.3109/2169140.2015.1047501. PMID: 26024447.
47. Mullon J, Giacoppe G, Clagett C, McCune D, Dillard T. Transfusions of polymerized bovine hemoglobin in a patient with severe autoimmune hemolytic anemia. N Engl J Med. 2000:342(22):1638–43. https://doi.org/10.1056/NEJM200006013422204. PMID: 10836875.
48. Fitzgerald MC, Chan JY, Ross AW, Liew SM, Butt WW, Baguley D, et al. A synthetic haemoglobin-based oxygen carrier and the reversal of cardiac hypoxia secondary to severe anaemia following trauma. Med J Aust. 2011;194(9):471–3. PMID: 21534906.

28

Development of Recombinant Hemoglobin-Based Oxygen Carriers-Somatogen: Studies and Lessons Learned

Kenneth Burhop

Background

Hemoglobin solutions have been investigated for their potential as blood substitutes for more than 70 years. Many of the very early experiments going back to those by Rabiner [1] and Amberson [2] demonstrated unwanted and unexpected side effects. The key side effects noted in these early experiments that involved infusion of the native hemoglobin tetramer included renal toxicity and hypertension [3]. In an effort to reduce, or ideally avoid these side effects, most of the early efforts focused their attention around new formulations that involved intermolecular linking of stroma-free hemoglobin, increasing the apparent molecular size of the hemoglobin products by such measures as chemical polymerization of the hemoglobin tetramer [4], PEGylation of the hemoglobin tetramer, or a combination of all of the above. The key idea of these approaches was to avoid dissociation of the tetrameric molecule in dimers [5, 6].

In almost all cases, the raw material for these products was derived from either human sources (outdated human RBCs) or animals, most typically derived from bovine sources. In these cases, and considering the large volumes that were would be potentially required to meet the projected demand, supply (vs potential demand) was always a concern. In addition, there was a significant concern regarding blood borne pathogens such as HIV or CJD with human blood and prions with bovine derived blood.

As a result of these constraints, the Somatogen team set out to design and develop a recombinant hemoglobin product (rHb1.1) that had three key features which were deemed desirable to function as a blood substitute or hemoglobin based oxygen carrier (HBOC) [7]. The design criteria included ensuring that the oxygen binding parameters of the new molecule be at least comparable to that of native erythrocyte associated hemoglobin and had a p50 in the range of 28 mmHg and favorable Hill coefficient. The second design criteria were that the molecule would be stable *in vivo* and not undergo dimerization creating dimers that were the cause of the early hemoglobin nephrotoxicity. The final design criteria were that in an effort to avoid disease concerns and to provide theoretically unlimited supply, that the product be produced by recombinant technologies and could be recovered as a soluble, properly folded hemoglobin molecule. Of course, production capabilities and cost were also considered.

The basic concept of utilizing recombinant technologies to produce cell-free hemoglobin solutions is not new and human hemoglobin synthesized in *Escherichia coli* [8] and *Saccharomyces cerevisiae* [9] have been investigated as potential oxygen-carrying blood substitutes. However, while this approach seemed straightforward on the surface, it turned out that these particular products could not be used as effective blood substitutes because they did not offload enough oxygen to the tissues (ie, in the absence of 2, 3-bisphosphoglycerate the oxygen affinity is too high) [10], they dissociated into alpha/beta dimers [11], and similar to other earlier hemoglobin solutions, they were rapidly cleared by the kidneys and caused renal toxicity [12, 13].

The breakthrough in this technological approach came when the team at Somatogen [7], successfully cloned, expressed and produced a human hemoglobin using an expression vector that contained one gene encoding a beta-globin with decreased oxygen affinity and one duplicated (2 copies), tandemly fused alpha-globin gene by a single codon encoding a glycine residue. The glycine codon was inserted between the two alpha chains and was arranged in tandem in the pSGE1.1E4 vector. This modification resulted in the expression of a di-alpha polypeptide where the fusion region between the two alpha chains contained the sequence Arg (141α1)GlyVal (1α2). Basically, the two alpha globin chains were fused with a glycine bridge between the C-terminal amino acid of one alpha globin chain and the N-terminal amino acid of the other alpha globin chain and the amino acid located in position 108 of both beta globin chains had been changed from a lysine to an asparagine.

K. Burhop (✉)
Fallbrook, CA, USA
e-mail: kenburhop@yahoo.com

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_28

In simple terms, Somatogen was the first ever to demonstrate that both human Hb chains could be expressed simultaneously and that a functioning Hb tetramer could be assembled in E. coli. Prior to this discovery, researchers were trying to express individual chains and then assemble them *in vitro* and add the heme group. This was clearly very inefficient. Until that time, the general consensus was that whole assembly could not be done.

As a result of the fusion of the two alpha-globin subunits, the product resulted in an *in vivo* decreased filtration by the kidney and a resultant increase in ½ life while not adversely affecting the protein (ie not affect the folding and assembly pathway of hemoglobin). Additional modifications were introduced to decrease the oxygen affinity (introduced an additional Asn-108β → Lys mutation mimicking the natural occurring Hb Presbyterian which had been previously reported to result in a hemoglobin with lower oxygen affinity in man). This substitution into rHb1.1 altered the oxygen-binding function in a manner that mimicked that of fresh blood and resulted in a right shift in the oxygen equilibrium curve such that approximately 30% more oxygen was released from rHb1.1 than from natural erythrocyte associated hemoglobin. The final modification to the molecule was a methionine substitution.

A number of experiments were conducted to characterize this new rHb1.1 engineered hemoglobin [14]. Xray crystallographic studies to the deoxy form of rHb1.1 at the 2-angstrom resolution level demonstrated that deoxy-rHb1.1 displayed the T state quarternary structure that was similar to the structure of deoxy native hemoglobin [15]. The substitution at the β108 as well as additional modifications did not significantly alter the deoxy T state conformation of the rH1.1 molecule when compared to native hemoglobin.

In order to function as a safe and effective HBOC, the product selected had to meet a number of critical design criteria including: favorable oxygen binding parameters, reasonable colloid oncotic pressure, did not activate the complement cascade and was non-immunogenic, did not produce renal toxicity, had to have an adequate *in vivo* half-life, must be stable *in-vivo* (ie, not undergo significant oxidation to methemoglobin or release heme or iron), and must not produce significant vasoconstriction.

As a result of the unique modifications in this genetically fused di-alpha hemoglobin this molecule (rHb1.1) could be produced in large amounts by simple microbial fermentation and purified without further modification in sufficient quantities to begin preclinical testing.

As summarized in a variety of different publications [13, 16, 17], the rH1.1 molecule met all of the original design criteria. In order to address the questions around oxygen delivery and efficacy, a variety of different exchange transfusion and hemorrhagic shock models were conducted in small and large animals. In these models, oxygen delivery was demonstrated by maintenance of high energy phosphates and intracellular pH as evaluated by ^{31}P NMR [18], by evaluation of oxygen maintenance and consumption in an ovine model of cardiopulmonary bypass and by repayment of oxygen debt.

Then to evaluate the questions around safety, studies were conducted in animals to assess a variety of different systems. To evaluate potential gastrointestinal effects of rHb1.1, experiments were conducted in a preclinical model designed to look at esophageal motility and the effects of rHb1.1 on lower esophageal sphincter (LES) function [13, 19, 20] as well as on the internal anal sphincter [21] and the sphincter of Oddi function in opossums since the anatomy and physiology of the opossum esophageal, large bowel, and other portions of the GI tract closely resemble man [22]. These experiments demonstrated that rHb1.1 caused a significant inhibition in swallowing and in relaxation of the LES.

The results of these and other studies suggested that these effects of rHb1.1 were mediated primarily due to the ability of rHb1.1, as with other acellular HBOCs, to scavenge nitric oxide. Unfortunately, similar effects were observed on blood pressure, intestinal blood flow and gut oxygenation in a rat model of hemorrhagic shock [23]. In a fixed pressure model of hemorrhagic shock and resuscitation, rHb1.1 increased MAP to 27% above the baseline values. Additional studies demonstrated that rHb1.1 demonstrated additional vasoconstrictive properties *in vivo* and *in vitro* that included increases in mean arterial pressure and systemic vascular resistance.

As a result of this extensive preclinical testing, the company was allowed to enter human clinical trials. From 1991 through 1993, Somatogen performed a series of trials to evaluate the safety and pharmaco-kinetics of rHb1.1 in 103 heathy, male volunteers (86 receiving rH1.1 and 17 receiving HSA as a control solution. The maximum dose reached in these early trials was 25 grams, and overall, the product was generally well tolerated [24].

In one early study, Optro (rHb1.1) was evaluated in 48 healthy male volunteers that ranged in age from 18–35 years of age [25]. In this study, the subjects were randomized to receive either an IV infusion of 5% rHb1.1 or an equal volume of 5% human serum albumin (HSA) over a time period of 1 to 2 h. The actual dose of the rHb1.1 ranged from 0.015 to 0.32 g/kg.

In this study, some of the subjects who received doses of 0.15 g/kg or greater were also given terbutaline, nifedipine, naloxone, nitroglycerin or glucagon. One subject required administration of epinephrine and diphenhydramine because of a reaction to the 0.015 g/kg of rHb1.1.

Following infusion of rHb1.1, there was no significant change in the urinary N-acetyl-β-glucosaminidase activity and no significant change in the serum creatine levels from the pre-infusion values. However, urinary hemoglobin was detected by dipstick in 56% of the subjects that received rHb1.1 and 36% of those receiving HSA.

There was an increase in mean arterial blood pressure and a decrease in heart rate observed in the subjects receiving rHb1.1. Systolic blood pressure increased immediately after the infusion of the rHb1.1 by approximately 5–50 mmHg, remained elevated until 6 to 8 h post infusion, and then ultimately decreased to baseline levels. These changes did not appear to be dose dependent.

From a pharmacokinetic modeling perspective, rHb1.1 concentration had a single compartment and the plasma clearance of rHb1.1 decreased and its half-life increased as the plasma concentration increased. The plasma clearance of rHb1.1 was dose related with a half-life of approximately 12 h at plasma concentrations of 5 mg/ml.

Overall, thirty-four of the rHb1.1 subjects had adverse reactions of some kind, with gastrointestinal tract effects being the most prevalent at doses of 0.15 g/kg or higher. Other symptoms reported included increases in lipase and amylase values, flu-like syndrome that included fever, chills and myalgias that appeared 4–8 h after the infusion and resolved in 12–24 h, Urticaria and/or itching developed in rHb1.1 subject but resolved with administration of IV diphenhydramine or Ibuprofen therapy. It is important to note that these "flu-like" symptoms were subsequently eliminated by a change in the manufacturing process of rHb1.1 which reduced all contaminating E. coli proteins and endotoxins to below detectable levels as measured by state-of-the-art analytical tools. Overall, there was no evidence of nephrotoxicity, immunogenicity, coagulation changes or any other significant clinical effects observed in these studies.

These early clinical trials demonstrated that rHb1.1 administration to awake volunteers resulted in mild to moderate upper gastrointestinal (GI) discomfort [25, 26]. Approximately half of the normal volunteers dosed with more than 5 g/kg of rHb1.1 experienced these transitory mild to moderate symptoms of dysphagia, vomiting and nausea. To better understand these effects and to translate what was learned in the preclinical studies, esophageal manometry studies were performed in male volunteers [27] that documented dysmotility.

The majority of these effects that included transient increases in blood pressure, dysphagia and amylase/lipase rise were all thought to be related to nitric oxide scavenging by the acellular hemoglobin product, which in turn, resulted in smooth muscle contraction in the vasculature as well as the smooth muscle of the esophagus.

rHb1.1 (Optro) was advanced into human clinical trials in patients. In a phase I/II trial, rHb1.1 was given to surgical patients for the first time in 1994 [28]. The study was a single-center, randomized, single-blind, dose-escalating, placebo-controlled trial in elective surgery patients. Surgeries were orthopedic, maxillofacial, plastic or urological and a total of 18 patients were enrolled (14 on rHb1.1 (Optro), and 4 on saline control. The dose was escalated to 25.6 g and the product was given after induction of general anesthesia when the patient was stable. Infusion was not based on physiological need.

There were no serious complications in this study and rHb1.1 was generally well tolerated. Most adverse events were similar in incidence and severity in both the rHb1.1 treated group and the saline control group and were thought to be due to the effects of anesthesia and surgery.

Transient increases in blood pressure were observed in the rHb1.1 group and resolved by 7 h post-infusion. Blood pressure rises did not correlate with dose and in those patients where therapeutic intervention was used, standard antihypertensive regimens were successful.

Five (36%) of 14 rHb1.1 and 1 (25%) of 4 saline-treated patients had elevations in serum amylase or lipase, but not both. Values generally peaked at 4–24 h and were normal at 24 h. No typical cases of acute pancreatitis were noted, though patients did report mild to moderate gastrointestinal symptoms, including nausea, vomiting, diarrhea and/or abdominal pain. These symptoms were also common in the saline group. Of note was that the magnitude of the enzyme rises appeared lower in this study than in previous volunteer trials.

Esophageal symptoms of epigastric pain and dysphagia were also notably absent as compared to prior volunteer experience. It is likely that the anesthetic agents used provided a dilatory stimulus to smooth muscle which to some degree countered the constrictive effect caused by nitric oxide scavenging.

From 1995 through about 1997, Somatogen conducted a number of phase II trials of rHb1.1 for indications including intra-operative blood replacement in 38 patients (25 receiving rHb1.1 and 13 control patients) [29], acute normovolemic hemodilution (10 patients with 7 rHb1.1 and 3 control) [30], surgical patients during general anesthesia (18 patients) [31] and enhanced ANH in cardiac surgery. These studies had dose-escalation designs. Overall, these studies demonstrated that rHb1.1 did result in some statistically significant changes including elevation in pancreatic enzymes, but the overall safety profile of the rHb1.1 appeared to be acceptable. There was no evidence of nephrotoxicity, immunogenicity or coagulation changes.

During this time of clinical investigation of rHb1.1, there were however multiple preclinical and clinical observations that all directed towards unwanted effects of the rHb1.1 with nitric oxide and the subsequent sequela. The "good news" was the as a result of the genetic engineering and recombinant expression approach, it allowed the scientists at Somatogen in close collaboration with Dr. John Olsen at Rice University to carefully alter the protein molecules to impart physiochemical and pharmacological properties that would be more optimal for therapeutic effects (eg, remove some of the unwanted effects seen such as increases in blood pressure, GI effects, etc.).

Molecular engineering was applied to further optimize the oxygen delivery of rHb1.1, modify the nitric oxide binding, increase the half-life/vascular retention, optimize the oncotic properties, and enhance the molecular stability. The most profound alterations were those that led to the development of a recombinant hemoglobin molecule which carried oxygen efficiently but exhibited reduced nitric oxide scavenging pharmacology [32–35]. Via this reduction, a large number of observed clinical "side effects" were also reduced or eliminated.

In contrast to random crosslinking chemistry which can result in a heterogeneous mixture of polymers with different sizes and function (unless additional molecular weight cutoff filtration is utilized), genetic engineering techniques allow you to modify the recombinant hemoglobin and provide a wide spectrum of approaches for producing larger molecular constructs that are homogeneous and well characterized.

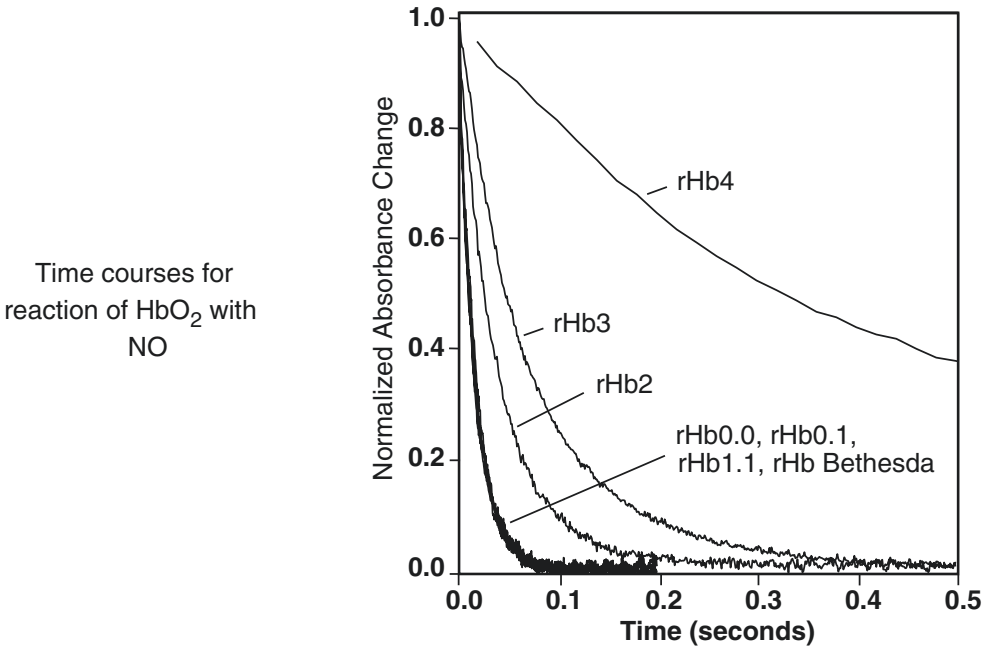
Simultaneous with this work, the Somatogen team in conjunction with Dr. John Olson at Rice University also investigated the concept that the oxidative reaction of nitric oxide with the bound oxygen of oxyhemoglobin may be of greater significance in nitric oxide scavenging than the simple binding of nitric oxide to the iron atom [32–35]. In order to investigate this, they made amino acid substitutions in the distal heme pocket of both subunits, thus lowering the reactivity of hemoglobin towards nitric oxide. They found that when larger, more bulky amino acids were inserted into the distal heme pocket, the reactivity of the resultant hemoglobin to nitric oxide was significantly reduced. Different modifications resulted in different molecules with different rate constants for nitric oxide reactivity, and in addition, altered the mean arterial pressure responses by rHb1.1 in direct relation to nitric oxide scavenging (Figs. 28.1, 28.2, and 28.3).

These data clearly demonstrated that oxygen delivery to arteriolar smooth muscle or parenchymal tissue cannot be the mechanism of the observed pressor effects of hemoglobin solutions as proposed by others [36]. The p50 of rHb Bethesda is low enough that this hemoglobin is basically incapable of delivering oxygen to tissues.

While a majority of side effects were reduced or eliminated with this approach, early studies in primates revealed that the new recombinant hemoglobin molecules still resulted in the production of cardiac lesions in select species of animals. The immediate answer to this issue was not known at that time.

During this same period of time, Baxter became aware of these cardiac lesions and had focused a great deal of effort on means to minimize/eliminate this effect [37]. However, and in the end, Baxter found that while polymerization or pegylation of the hemoglobin molecule could reduce the incidence and severity of the cardiac lesions, these approaches could not eliminate the lesions in sensitive species (eg, pigs, rhesus monkeys, etc.). Preclinical evidence suggested that somehow, nitric oxide binding may be involved in the development of the heart lesions and was likely responsible for the vasoactivity seen with DCLHb. Baxter realized early on that it would need to change the inherent properties of the native hemoglobin molecule via recombinant technology to ultimately be successful.

Therefore in 1998 Baxter stopped all development of their first generation hemoglobin products (eg, DCLHb) and



Doherty et al., Nature Biotechnol. 16: 672, 1998

Fig. 28.1 The NO binding kinetics of hemoglobin can be varied

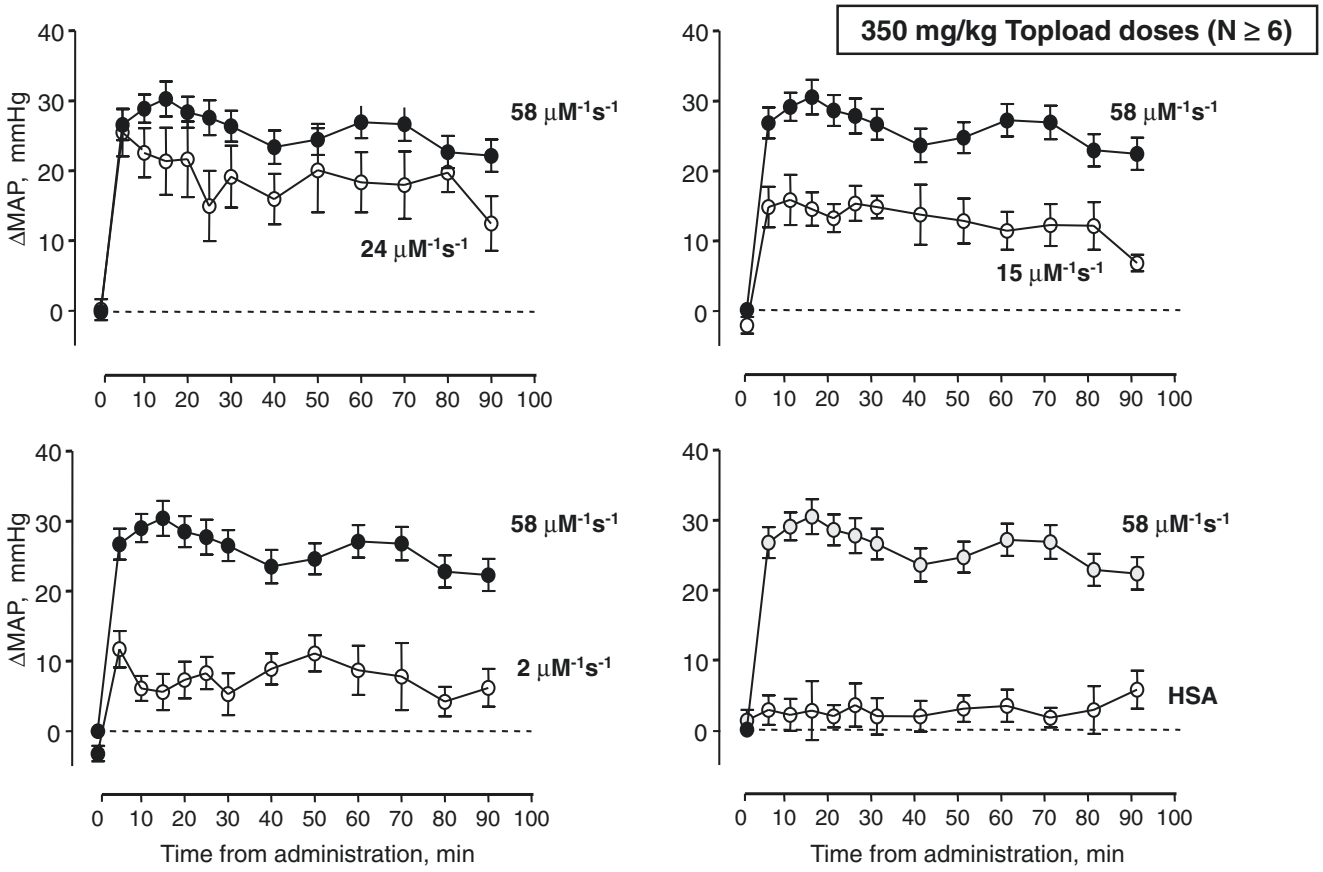
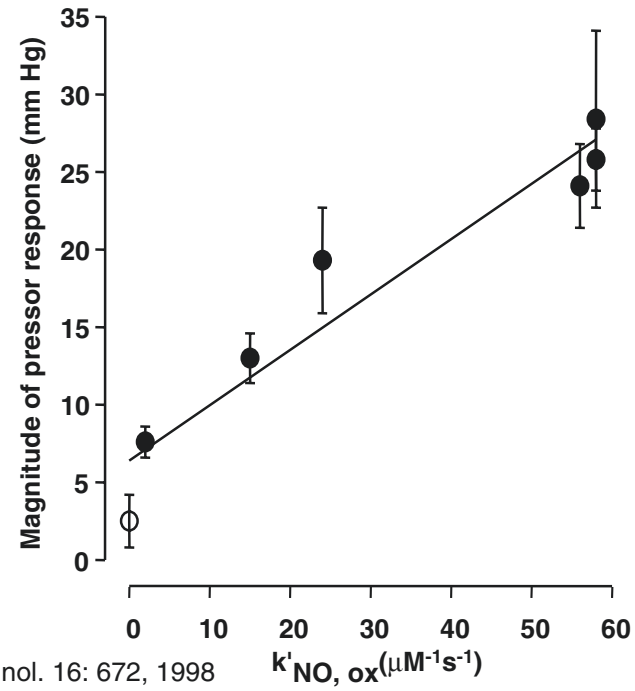


Fig. 28.2 The pressor responses in rats correlates with the NO scavenging rate

Fig. 28.3 The pressor responses directly correlate with NO scavenging rate



Doherty et al., Nature Biotechnol. 16: 672, 1998

acquired Somotagen for its recombinant hemoglobin technology. The purchase of Somatogen along with their strong intellectual property position provided Baxter the "freedom" to move forward with the development of a second generation recombinant hemoglobin technology.

Second Generation Recombinant Hemoglobin Development

Significant progress was made in developing a safe and effective hemoglobin-based oxygen carrier (HBOC) for clinical use. Many of the issues encountered in the very early attempts to develop these products were addressed with the HBOC products being tested at that time (late 1990s). Early "stroma reduced" hemoglobin solutions had primary safety concerns: short intravascular half-life, renal toxicity, residual red blood cell stroma and endotoxin contamination. Each of these issues were resolved through stabilization of the hemoglobin tetramer by chemical and/or recombinant means, enhancing the size of the hemoglobin product using polymerization and derivatization technologies, and using advanced techniques of protein purification and process engineering.

As discussed earlier, the strong interaction of nitric oxide (NO) with hemoglobin had been known for more than 30 years. However, during the development of these different hemoglobin products for clinical use, nitric oxide was found to be a ubiquitous and potent chemical messenger throughout the body. In the first-generation hemoglobin program at Baxter it was believed that the increase in arterial blood pressure seen following infusion of DCLHb would not pose a significant problem. In fact, there was considerable evidence suggesting that this pressor effect could be used to a clinical advantage, and that HBOCs, could be used as pharmacologic agents. However, after review of Baxter's clinical trial results and additional preclinical study results, it appeared that the interaction of hemoglobin with nitric oxide and the physiologic and pathophysiologic consequences of this interaction may have been responsible for many of the adverse effects observed with the first generation of purified and modified hemoglobin solutions that were investigated in the clinic in 1990s.

Therefore, Baxter made a strategic program decision to stop development of its first generation hemoglobin products (ie, DCLHb) and Somatogen's first generation product (ie, rHb1.1) which possessed significant nitric oxide reactivity similar to that of native hemoglobin, and to develop a second generation hemoglobin product with reduced nitric oxide scavenging.

At that time, Baxter formulated a list of "potential issues" that were associated with the first-generation hemoglobin-based oxygen carriers as part of its termination of the first-generation hemoglobin programs. This list included the following:

- Vasoactivity/increase in arterial blood pressure
- Extravasation of hemoglobin
- Relatively short half-life
- Cardiac lesions
- Gastrointestinal dysmotility
- Increase in serum enzymes
- Oxidant stress
- Effects on the pancreas
- Hb/endotoxin interaction

This list of "potential issues" formulated for first generation hemoglobin products was the primary tool utilized to develop the preclinical study plan for the final selected product.

A target product profile (TPP) was generated and formed the basis for making a decision when or if to enter into clinical studies in man. Some other goals that were considered in the development of the preclinical study plan were to ensure that: (1) animal models utilized in the preclinical testing were relevant to the ultimate clinical use of the second generation product (attempt to maintain a strong preclinical-clinical linkage), (2) similar doses and protocols were utilized in a number of different animal species and models so that data could be more easily compared across all of the testing, (3) the new second generation molecule was tested in more than one animal species, (4) the most sensitive and appropriate models previously identified with first generation hemoglobin products tested were utilized, (5) key issues identified in the FDA "Points to Consider" document (CBER, 1991) as well as discussions held during the pre-IND meetings with the FDA were addressed, and (6) knowledge gained from the preclinical testing of first generation products was utilized to formulate rational and predictive testing plans for the new second generation product. Key safety studies were performed under strict GLP compliance. Simultaneously, a wide variety of other supportive safety and efficacy studies were either performed within the Hemoglobin Therapeutics Program at Baxter or at leading academic universities around the world with thought leaders identified in specific fields and with specific published models [38–41]. Taken together, these represented a comprehensive examination of the preclinical safety and efficacy of the new second generation hemoglobin molecule.

Over the next several years, over 535 different hemoglobin variants were produced utilizing site directed mutagenesis of the recombinant hemoglobin molecule. A variety of chemical cross-linking or 'decoration" (eg, PEGs) agents were also examined. These various products were examined utilizing a tiered preclinical testing approach beginning with chemical testing, following a pre-defined series *of in-vitro* models and tests, followed by testing of the most promising

products in pre-defined small animal models specifically developed to assess the key "potential issues" list identified in the upfront process. Then those molecules that were the most promising and passed all of these hurdles were tested in another set of pre-defined, sensitive large animal models, with only a select few making it to the final primate testing program.

The final candidate molecule was designated as rHb2.0 for Injection. It was a second-generation recombinant human hemoglobin solution that was expressed in *E. coli*. The monomer contained the following site directed mutations: aL29W, aH58Q, bV67W, and bT87Q. The final rHb2.0 for Injection product was then further polymerized and derivatized using a bi-functional BMA-PEG. Similar to rHb1.1, the α chains were fused to prevent dissociation and serval amino acid substitution were made in the distal heme pocket by site-directed mutagenesis to reduce the rate of NO scavenging. The hemoglobin product was contained in a matrix of 9 mM N-acetyl-L-cysteine, electrolytes and EDTA and formulated at 100 g/l (Fig. 28.4).

The list of actual testing and outcomes is far to large to review in detail in this review, but included tests such as:

- Models of systemic and pulmonary vasoactivity (eg, assessment of CV parameters and measurement of regional blood flow in rat and swine models, tissue oxygenation models, microcirculatory studies in hamsters, isolated human vascular segments; isolated rat lungs)
- Gatrointestinal motility (eg, gastric emptying in rats)
- Oxygenation and effectiveness (eg, uncontrolled hemorrhage and resuscitation in rats and swine, ability to reverse oxygen supply dependence in rats, effects in isovolemic exchange models)
- ADME (eg, pK in rats and rhesus monkeys following single and repeat doses)
- Biodistribution (eg, pK, distribution and excretion of radioactivity following single IV dosing in rats; L/P ratio of extravasation in rats; inhibitory potential on human hepatic microsomal cytochrome p450 isoenzyme activities and in the rat)
- General toxicity (eg, single dose toxicity study in rats, Rhesus monkeys and repeat dose testing in rats and Rhesus monkeys)
- Immunology (eg, 15-week immunogenicity study of repeated IV infusion in Rhesus monkeys, 10 week study of repeated administration in mice)
- Organ specific toxicity (eg, evaluation of effect on serum pancreatic enzymes in rats and in Australian Possums; assessment of renal function in the rat)
- Body system specific toxicity (eg, toxicity in mice given rHb2.0 and lipopolysaccharide*; in vitro* concentration-response on coagulation in human blood and on platelet aggregation in human blood, effect on complement activation in human plasma, effect on Rouleaux formation in human blood, red blood cell aggregation and regional ischemia/reperfusion injury in the cerebrum of the rat; effect on plasma isoprostanes and histopathology in diabetic BBZDR rats)

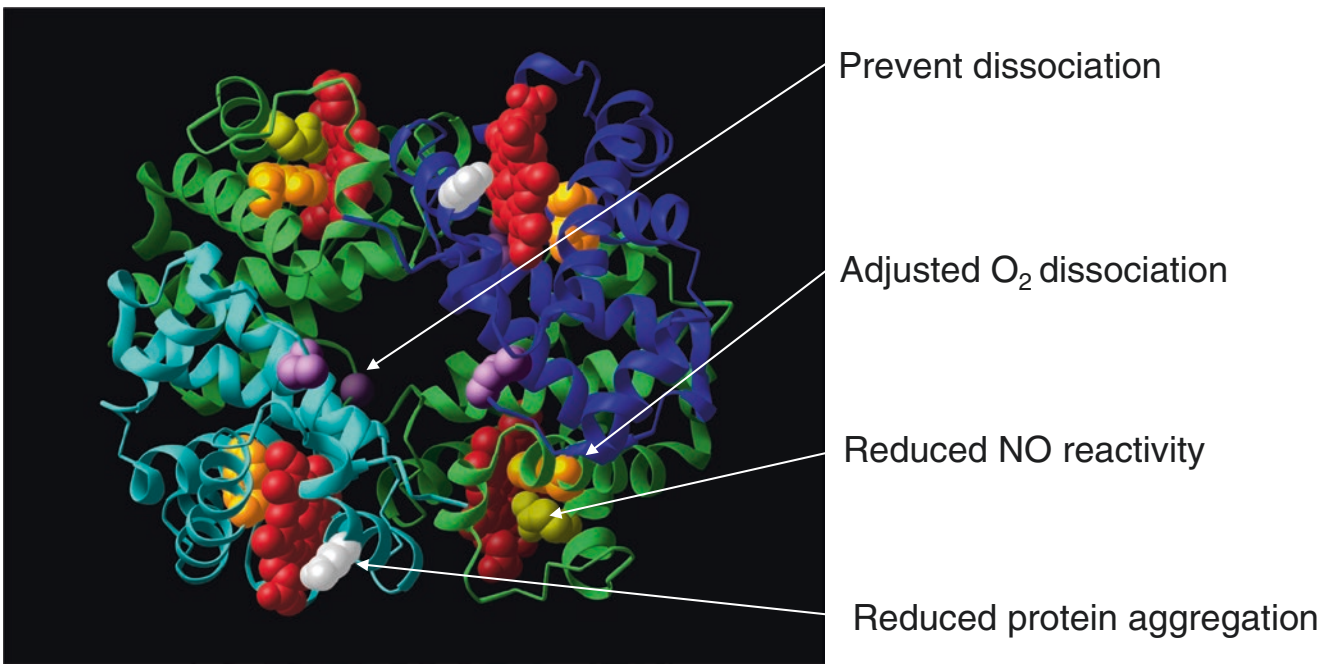


Fig. 28.4 Baxter's rHb2.0 molecule was genetically engineered using recombinant technology

In the end, no-effects dose tables were created for all of the various tests of rHb2.0. A key finding was that there was no increase in systemic arterial blood pressure and no change in systemic vascular resistance seen in any animal models. The vacuolation of histiocytes/macrophages was a consistent finding across all of the species studied. The cause of the cytoplasmic vacuolation is unknown, but the pattern of vacuolation in cells capable of phagocytosis suggested that the clearance or degradation of the rHb2.0 was involved, and therefore, it may have been due to the presence of the PEG. Notably the cytoplasmic vacuolation was not accompanied by evidence of inflammation or cellular degeneration and the changes were reversible. Thus, it was determined that this finding had few, if any, adverse consequences clinically.

Importantly, myocardial lesions related to rHb2.0 for Injection were not observed in either the primate studies or in the swine cardiovascular function studies. In the immunogenicity studies in rhesus monkeys and mice, increased antibody levels reactive to rHb2.0 were observed. The fact that there were non-responders in every dose group suggested that rHb2.0 for Injection was only mildly to moderately immunogenic in these animal models. The antibodies were specific to rHb2.0 and did not cross-react with the monomer of rHb2.0 for Injection. Furthermore, these antibody responses were not associated with any toxicological findings to indicate an immune mediated event, disorder or disease.

Therefore, and overall, the results of extensive preclinical testing demonstrated that rHb2.0 reduced or eliminated all of the issues found during the preclinical and clinical development of first generation HBOCs. The toxicity studies suggested that rHb2.0 for Injection was justified to move forward into clinical testing.

The rHb2.0 for Injection was then tested in April 2001 in a blinded, single dose escalation study in normal volunteers. Four subjects received 0.0066 g/kg of rHb2.0 and two subjects received 0.0066 g/kg of HSA. A series of immunologic studies were conducted evaluating complement activation (C3a levels), cytokines (TNF-a, IL-1b, IL-6) and antibody response (IgG and IgM against ECP and rHb2.0).

There was no observed increase in blood pressure, but unfortunately, and within about 15–30 minutes following start of infusion of the lowest dose of the product, a physiological reaction was observed that was associated with a tightness in the chest and dyspnea and was later confirmed to be a result of complement activation (ie, neutropenic response and a significant increase in plasma C3a that peaked at 30 minutes and returned to normal by 24 h post infusion). The phase 1 studies were halted by Baxter.

The next several years were spent trying to understand the cause of this immunologic reaction in humans and to determine if it was via the classical, alternative or lectin pathway.

IgM levels showed an increase at day 7, and remained elevated at 1, 3 and 6 months post-infusion. IgG levels increased at day 14, and remained elevated at 1, 3 and 6 months post-infusion. The cause of complement activation and antibody response was unlikely due to a pyrogenic response since no elevation of cytokines levels were observed in the subjects. The presence of pre-existing antibodies suggested complement activation via the classical pathway. Assessment of the lectin pathway and inhibition of various complement activation pathways was performed by Dr. Greg Stahl, at Harvard University. Dr. Stahl tested the clinical lot of rHb2.0 with and without specific monoclonal antibodies. C3 deposition was decreased with anti-C2 but not with anti-MBL and anti-factor D.

Detailed examination of the rHb2.0 lot with SEC of the rHb2.0 on a Superose 6 Column, did identify a high molecular weight peak of rHb2.0 in the clinical batch with about a 16 min retention time. Immunological testing demonstrated cross-reactivity of IgM antibodies to the clinical lot of rHb2.0 but not to rHb2.0 monomer or to stroma free hemoglobin. Cross-Reactivity of IgM antibodies to another lot of rHb2.0 also showed cross-reactivity and this lot had NAC used in the quench and formulation but there was no high MW fraction. In contrast, when a different (new) lot of rHb2.0 was prepared with no NAC used in the quench or formulation and with no high MW fraction there was no cross-reactivity of IgM antibodies. Therefore, these observations suggested that epitope for the pre-existing antibodies was to N-acetylcysteinyl succinimide (ring open form).

The overall conclusion from the investigational studies on rHb2.0 for Injection were that:

- Some percent of population of all species tested (ie, humans, sheep, mice, and monkeys) have pre-existing antibodies to something in the particular quench agent (NAC) that was used to prepare rHb2.0 NAC-(ie, likely Succinyl-PEG)
- This "may" relate back to some previous history of infection to agents like mycoplasma, strep, *E. coli*, Haemophilus influenzae, etc. [42]
- The complement activation in humans and in sheep was the result of the pre-existing antibodies forming a complex with rHb2.0 (especially the larger MW components), and triggering the classical pathway
- Baxter didn't know if there was another delayed response that was hidden or not, because it would have been masked by the first and stronger response seen in man

As a direct result of this extensive testing, improvements were made to the rHb2.0. These improvements included:

- Reducing the high MW component by ultrafiltration of the BMA-PEG

- Changing the quench reagent used following BMA-PEG reactions with rHb2.0 monomer from N-Acetyl-L-cysteine (NAC) to 2-Mercaptoethylamine (MEA). MEA is also known as cysteamine. It is used as a therapeutic agent in the treatment of cystinosis. It functions to remove cystine from peripheral tissues, promote glutamine biosynthesis and retention of nitrogen in the amino acid reservoir *in vivo*. Of all thiol agents evaluated, MEA reacted fastest with maleamic acid groups. Studies showed that the products formed by MEA quenching were not recognized by pre-existing antibodies. The amount of residual cysteamine in a 2 L dose of recombinant hemoglobin was 535-fold less than the intravenous LD50 in the rabbit, the most sensitive species
- Changed the formulation from 9 mM NAC to 2 mM ascorbate

This new product was designated rHb2.1. Endotoxin contamination was ruled out as the product passed the LAL test and rabbit pyrogen tests. Additional downstream processing was also incorporated with the addition of lipid-removal agent to remove any residual lipids that might be present and below level of detection in the product. A more sensitive test was also developed utilizing isolated human macrophages and measurement of IL-6 and the new rHb2.1 product passed these tests.

Human population screening for pre-existing antibodies to rHb2.1 was conducted to examine the potential to generated IgG and IgM antibodies to the rHb molecules (ie, rHb2.0 and rHb2.1). This testing was conducted utilizing approximately 500 sera samples of human serum from across the United States and collected from the four basic regions across the United States (eg, North, South, East and West regions). Approximately 80 samples were also tested for in the C3 deposition assay. 123 of 538 samples tested positive for pre-existing IgG antibodies to rHb2.0 and only 2 of 538 samples tested positive for pre-existing antibodies to rHb2.1. 253 of 539 samples tested positive for pre-existing IgM antibodies to rHb2.0 and 3 of 539 human sera samples tested positive for pre-existing IgM antibodies to rHb2.1. None of the samples tested positive for pre-existing IgG or IgM antibodies to rHb2.1 SFHb.

The rHb2.1 product was then tested in rhesus monkeys and no immunologic response could be detected. In this study, three monkeys were dosed with 2.7 g/kg HSA, five monkeys were dosed with 2 g/kg rHb2.0 and five monkeys were dosed initially with 2 g/kg with rHb2.1 and 4 days later dosed again with 2 g/kg of rHb2.0. There was no complement response (C3a) in the monkeys receiving HSA, there was an increase in C3a in monkeys dosed with rHb2.0 (as soon as 5 minutes post-injection), and there was no complement response in monkeys dosed with rHb2.1. In this cross-over study, 4 days later IgM antibody levels were again tested in the monkeys that had previously received rHb2.1. Levels were found to be at baseline levels. However, 4 h post subsequent dosing of rHb2.0 in these monkeys, there was a strong IgM response. Three additional studies were also conducted to examine the complement response in Rhesus and Cynomologus monkeys. Overall, none of the 20 monkeys dosed with rHb2.1 elicited a C3a response after the first Dose. One out five (1/5) monkeys in the immunogenicity study (dosed every 2 weeks for 5 doses) elicited a complement response after the third dose. This monkey also elicited an antibody response (IgG after the first dose), however, the antibodies cross-reacted to SFHb. The antibodies did not cross-react to the PEG or PEG-NAC or PEG-MEA. Two additional monkeys elicited an IgG antibody response after the fourth dose (endpoint titer 500). After a 3 month recovery period, the monkeys in the immunogenicity study were re-challenged (sixth dose) and none of the monkeys elicited a complement response.

The rHb2.1 product was also tested in an ovine model of complement activation with specific reagents developed to measure ovine C3a and ovine C5a and no complement reaction was observed. After several expert panel meetings with experts from around the world, and after extensive testing by world experts in the lectin pathway, there was no evidence of complement activation via this pathway either.

Therefore, and after additional downstream process steps had been incorporated, and since all preclinical *in vivo* and *in vitro* tests appeared negative, in October 2002 Baxter approached the FDA with the data and were allowed to restart clinical testing in human volunteers at low doses of rHb2.1.

The phase 1 testing of the rHb2.1 was to include doses of 0.005, 0.02, 0.05, 0.1, 0.25, 0.5 and 1 g/kg. There number of proposed subjects were n = 4 for rHb2.1 and n = 2 for HSA. Initially, very low dose administration was conducted to observe any serious, unexpected adverse outcomes (four fold increase in dose with the next dose group). Progressive dose escalation (2–2.5 fold increases) from 0.005 to 1.0 g/kg (with exception of what is noted previously). Dosing then progressed to the fourth dose group in the rHb2.1 Phase I clinical trial. Twenty-four subjects were dosed in the rHb2.1 Phase I clinical trial (16 with rHb2.1, 8 with HSA). Eighteen subjects (12 rHb2.1, 6 HSA) were administered rHb2.1 through dose Group #3 and the product was judged as safe and well tolerated.

In terms of general clinical outcomes, the team successfully achieved triple blinding (subject, investigator, monitor) of study drug treatment assignment through study drug administrations in the Phase I trial. The study provided a basis for "proof of principle" of the metabolic advantage of second generation rHb over first generation Hb, based on NO scavenging modifications, size modifications, etc. There was no evidence of any hemoglobinuria with rHb2.1, in contrast

to findings with the first gen product. There were increases in serum total bilirubin (expected per metabolism pathway) but no reports of skin discoloration (yellow) (jaundice) through doses up to 0.1 g/kg of rHb2.1.

There were no increases in systolic or diastolic blood pressure in humans as a result of rHb2.1 administration. There were no significant increases in C3a seen in the four subjects dosed with 0.005 g/kg of rHb2.1, none in the four subjects dosed with 0.02 g/kg of rHb2.1, and none in the four subjects doses with 0.05 g/kg of rHb2.1. However, there was a delayed increase in C3a seen at 8 h post-dosing in subjects in the fourth dosing group that was dosed with 0.1 g/kg of rHb2.1. This response did not appear to be related to antibody formation.

The obvious question was "what's the cause of the delayed complement activation response in the human volunteers after this particular dose of rHb2.1"?

Additional testing of the clinical response examining the complement response by Dr. P. Giclas, Director, Complement Laboratory, National Jewish Medical and Research Center, Denver, CO demonstrated a Bb response at 8 h following dosing of the 0.1 g/kg of rHb2.1 with no consistent change compared to the HSA controls in the C4a response or Factor H response.

Overall, the clinical response occurred several hours after infusion. In contrast to the phase I clinical trial results with rHb2.0, the complement response was not immediate, but rather, was late (i.e., around 8 h post-dosing). For all four dose groups, there was no evidence of pre-existing antibodies (IgM or IgG) to rHb2.1 and to ECPs (in contrast to rHb2.0 in which there were pre-existing antibodies to the product). There was no evidence of de novo antibody formation following infusion of rHb2.1 for Injection. Complement activation appeared to by means of the alternative pathway. Based on data collected, Baxter believed that the adverse response was due to higher than desirable levels of some sort of pyrogen in the product as detected with a sensitive IL-6 assay that Baxter had developed. It is likely that these observations were masked/missed in the original phase 1 trial of rHb2.0 due to the higher immediate complement response.

There was also a slight tachycardia response and some cytokine elevation in the subjects. This was difficult to explain since all lots of rHb2.1 produced for clinical trials passed the rabbit pyrogen test (both monomers and the final product), all lots had LAL values below the established release limits (<0.25 EU/mL), and up until that time, the *ex-vivo* PBMC IL-6 assay was the most sensitive assay identified for detection of this "factor". The Baxter team was aware that the rHb2.1 had "some" level of IL-6 response, but there were no benchmarks. Therefore, the only alternative was to use the animal data to set preliminary limits. Unfortunately, these limits appeared to turn out to be too high for humans (most sensitive species).

Following this, additional process improvements were examined to attempt to decrease the non-endotoxin pyrogen contamination. The team examined things like a Zinc column, triton wash Q column, guard column and reduced load to 75%. All of these steps combined did apparently reduce the levels of this "factor". However, the dilemma was what test system could be utilized to evaluate the progress on the problem?

As a final attempt, a lipid removal agent (LRA) was evaluated. LRA is a synthetic or semi-synthetic calcium silicate that has remarkable affinity for lipids and lipid-like materials. Treatment of rHb2.0 Q load reduced the endotoxin level by 3 to 4 logs and allowed production of non-pyrogenic Q Pools (by IL-6). Through an extraordinary effort involving process development, manufacturing and maintenance personnel, the LRA treatment was scaled up and implemented in GMP operations in manufacturing.

Addition of LRA to the Q LOAD significantly reduced the pyrogen as measured by flow TNF-alpha, IL-6, and rabbit pyrogen assays. Addition of LRA also appeared to remove ECPs normally removed by the Triton wash step.

As noted, the LRA was introduced into the most current manufacturing process essentially like a "filter" on the Q load material. Once sufficient material was produced for Phase I clinical trials, the company performed a "mini-shutdown" of the pilot plant to implement several additional changes: (1) Complete formal addition of LRA into the process, (2) Complete a few facility upgrades (e.g., improve the entry area to purification suites), (3) Complete annual required media fills, validations, etc. and (4) implement several additional PD changes that were identified.

The clinical plan was then to restart the rHb2.1 Phase I study at the third dose level (0.05 g/kg) that was successfully and safely administered in the initial Phase I rHb2.1 study. The idea was to target to achieve 0.25 g/kg dose in Phase I, fifth dose group, to support proceeding to Phase II clinical trials. 0.25 g/kg would be first dose in Phase II. If possible, and if it was not possible to reach the "limit of tolerability" in normal volunteers, the plan was to attempt to complete the 6th (0.5 g/kg) and 7th (1.0 g/kg) dose group in the phase I clinical trial.

Unfortunately, and similar to the first human testing of the rHb2.0 molecule, the newly formulated rHb2.1 product produced a delayed complement activation response in humans. No antibodies were observed at any point in time and pyrogen levels were equivalent to control levels. No animal models ever predicted this final response.

At that point in time, and in light of the fact that there were no *in vitro* tests and no *in vivo* animal models that could be identified to understand the cause of this reaction and/or be able to demonstrate that the adverse effect was removed, in July 2003 the decision was made to terminate all activities on development of an HBOC at Baxter. As part of larger cor-

porate restructuring and cost reduction program, the Boulder site was closed and all physical assets were sold.

To this day, and to the authors best knowledge, there is no definitive explanation for these effects, which is extremely unfortunate as this approach with this class of recombinant hemoglobin products represented a potentially valuable approach to producing a commercially viable HBOC solution.

Since the termination of this program, work has continued in a variety of laboratories around the world to continue to investigate the development and subsequent clinical use of hemoglobin solutions, but to date, and unfortunately, there has been no new HBOC that has entered human clinical trials.

The following are some hypotheses of what may have been wrong with rHb2.1 for Injection:

1. The crosslinker, BMA-PEG/MEA, produced some epitope recognized as foreign by the body and a complement response was triggered
2. The glycine linker in the basic monomer was immunogenic
3. Aggregates may have formed
4. The end terminal methionines may be the cause (40% methylated)
5. T87Q mutation to reduce aggregation was involved
6. Heme pocket mutations for reduced nitric oxide were responsible for the observed effects
7. The product oxidized very rapidly *in vitro* (potentially less stable)
8. Some low level of contaminant (e.g., *E coli* proteins) that was undetectable remained in the product
9. Something about some isoform of rHb2.1 that resembles something from *E. coli* that is recognized by humans as foreign
10. Some unique amino acid sequence that forms during metabolism and breakdown of the polymer/monomer was responsible for the observed effects

Summary

While the final outcome from several decades of scientific research and product development was certainly not optimal or what had been hoped for, this comprehensive development effort made a number of significant contributions to the field of hemoglobin-based oxygen carriers. Baxter (Somatogen) scientists demonstrated for the first time that that both human Hb chains could be expressed simultaneously and a functioning Hb tetramer would be assembled in E. coli and that they could produce recombinant hemoglobin in *E.coli* at high yield (10+ g/L) with very purity. Baxter (Somatogen) demonstrated that it could manufacture recombinant hemoglobin on a large scale under GMP conditions at the 50,000 liter fermentor scale (rHb1.1) and at the 1500 liter fermentor scale (rHb2.0). Baxter scientists demonstrated that they could change the basic structure of a recombinant hemoglobin molecule via site directed mutagenesis, and by doing so, control the nitric oxide binding rate and oxygen dissociation. Unfortunately, there still remains a number of significant hurdles that would need to be overcome in order to enable full product development and commercialization of a recombinant hemoglobin based oxygen carrier, with the biggest hurdle likely being identification of an appropriate animal model (other than humans) in which to test new candidate products.

Key Points

- Baxter (Somatogen) scientists demonstrated that that both human Hb chains could be expressed simultaneously and a functioning Hb tetramer would be assembled in E. coli
- Baxter (Somatogen) demonstrated that they could produce recombinant hemoglobin in E.coli at high yield (10+ g/L) with very high purity.
- Baxter (Somatogen) demonstrated that it could manufacture recombinant hemoglobin on a large scale under GMP conditions at the 50,000 liter fermentor scale (rHb1.1) and at the 1500 liter fermentor scale (rHb2.0).
- Baxter scientists demonstrated that they could change the basic structure of a recombinant hemoglobin molecule via site directed mutagenesis, and by doing so, control the nitric oxide binding rate and oxygen dissociation.

References

1. Rabiner SF, Helbert JR, Lopas H, Friedman LH. Evaluation of a stroma-free hemoglobin solution for use as a plasma expander. J Experiment Med. 1967;126(6):1127–42.
2. Amberson W, Jennings JJ, Rhode M. Clinical experience with hemoglobin-saline solutions. J. Appl Physiol. 1949;1:469–89.
3. Sunder-Plassmann RD, Seifert J, Jesch F, Messmer K. Stromafree haemoglobin solution as a blood replacement fluid actual state and problems. Europ J Intensive Care Med. 1975;1:37–42.
4. Moss GS, Gould SA, Sehgal LR, Sehgal HL, Rosen AL. Hemoglobin solution from tetramer to polymer, Biomat Artif Cells Artif Organs. 1988;16:57–69.
5. Blood substitutes: present and future perspectives. Tsuchida E (ed). Elsevier; 1998.
6. Blood Substitutes, Winslow RM (ed). Elsevier; 2006.
7. Looker D, Abbott-Brown D, Cozart P, Durfee S, Hoffman S, Mathews AJ, Miller-Roehrich J, Shoemaker S, Trimble S, Fermi G, Komiyama NH, Nagai K, Stetler GL. A human recombinant haemoglobin designed for use as a blood substitute. Nature. 1992;356:258–60.
8. Hoffman SJ, Looker DL, Roehrich JM, Cozart PE, Durfee SL, Tedesco JL, Stetler GL. Proc Natn Acad Sci USA. 1990;87:8521–5.
9. Wagenbach M, O'Rourke K, Vitez L, Wieczorek A, Hoffman S, Durfee S, Tedesco J, Stetler G. Synthesis of Wild Type and

Mutant Human Hemoglobins in *Saccharomyces cerevisiae*. Nature Biotechnol. 1991;9:57–61.
10. Benesch R, Benesch RE. Biochem Biophys Res Commun. 1967;26:162–7.
11. Ackers GK, Halvorson HR. Proc nstl Acad Sci USA. 1974;71:4312–6.
12. Bunn HF, Esham W, Bull RJ. J Exp Med. 1969;129:909–23.
13. Zuckerman SH, Doyle MP, Gorczynski R, Rosenthal GJ. Preclinical biology of recombinant human hemoglobin rHb1.1. Art Cells Blood Substitutes Biotechnol. 1998;26(3):231–57.
14. Nelson DJ. In: RM Winslow (ed). DCLHb and rHb1.1. Elsevier; 2006. p. 399–414.
15. Kroeger KS, Kundrot CE. Structures of a hemoglobin-based blood substitute: insights into the function of allosteric proteins. Structure. 1997;15:227–37.
16. Siegel JH, Fabian M, Smith JA, Constantino D. Use of recombinant hemoglobin solution in reversing lethal hemorrhagic hypovolemic oxygen debt shock. J Trauma. 1997;42:199–212.
17. Doyle MP, Fink N, Rosenthal GJ, Gorczynski R. Efficacy of recombinant human hemoglobin to restore oxygen consumption following hemorrhage. Artif Cells Blood Substit Immobilizat Biotechnol. 1996;24:330a.
18. Sillerud L. O, A. Caprihan, N Berton and Gary J Rosenthal. Efficacy of recombinant human Hb by ^{31}P-NMR during isovolemic exchange transfusion. J Appl Physiol. 1999;86(3):887–94.
19. Chakder S, Rosenthal GJ, Rattan S. *In vivo* and in vitro influence of human recombinant hemoglobin on esophageal function. Am J Physiol. 1995;268:G443–50.
20. Conklin JL, Murray J, Ledlow A, Clark E, Hayek B, Picken H, Rosenthal G. Effects of recombinant human hemoglobin on motor functions of the opossum esophagus. J Pharmacol Exp Ther. 1995;273:762–7.
21. Rattan S, Rosenthal GJ, Chakder S. Human recombinant hemoglobin (RHb1.1) inhibits nonadrenergic noncholinergic(NANC) nerve-mediated relaxation of internal anal sphincter. J Pharmacol Exp Ther. 1995;272:1211–6.
22. Cullen JJ, Conklin JL, Murray J, Ledlow A, Rosenthal G. Effects of recombinant human hemoglobin on opossum sphincter of Oddi motor function *in vivo* and *in vitro*. Dig Dis Sci. 1996;41:289–94.
23. Raat NJH, Liu JF, Doyle MP, Burhop KE, Klein J, Ince C. Effects of recombinant-hemoglobin solutions rHb2.0 and rHb1.1 on blood pressure, intestinal blood flow, and gut oxygenation in a rat model of hemorrhagic shock. J Lab Clin Med. 2005;145:21–32.
24. Freytag JW, Caspari RF, Gorczynski RJ. Recent progress in the development of recombinant human hemoglobin (rHb1.1) as an oxygen therapeutic. In: Tsuchida E, editor. Blood Substitutes – Present and future perspectives. Elsevier Science S.A.; 1998.
25. Viele MK, Weiskopf RB, Fisher D. Recombinant human hemoglobin does not affect renal function in humans: Analysis of safety and pharmacokinetics. Anesthesiology. 1997;86:848–58.
26. Gerber MJ, Stetler GL, Templeton D. Recombinant human hemoglobin designed for use as a oxygen delivering therapeutic. In: Tsuchida E, editor. Artificial Red Cells. John Wiley & Sons Ltd; 1995. p. 187–97.
27. Murray JA, Ledlow A, Launspach J, Evans D, Loveday M, Conklin JL. The effects of recombinant human hemoglobin on esophageal motor function in humans. Gastroenterol. 1995;109:1241–8.
28. Lind GH, Stanley T, Caspari RF, Brown EB. A double-blind study to evaluate the safety of recombinant human hemoglobin (rHb1.1) in surgery patients under general anesthesia. Presented in abstract form: the 36 annual meeting of the American Society of Hematology. Blood. 1994;84(10 Suppl. 1):530A.
29. Leone BJ, Chuey C, Gleason D, Steele SM, Greengrass R, Capsari RF, Scanga R. Can recombinant human hemoglobin make ANH more effective? An initial feasibility and safety study. Anesth Analg. 1996;82/2S:S274.
30. Lessen R, Williams M, Seltzer J, Lessin J, Marr A, Guvakov D, Imbing M, Solanski D, Grugan K, Smith D, Kittle D, Lawson N, Caspari R, Loveday M. A safety study of recombinant human hemoglobin for intraoperative transfusion therapy. Anesth Analg. 1996;82/2S:S275.
31. Hayes JK, Stanley TH, Lind GH, East K, Smith B, Kessler K. A double-blind study to evaluate the safety of recombinant human hemoglobin in surgical patients during general anesthesia. J Cardiothoracic Vascul Anesthesia. 2001;15(5):593–602.
32. Eich RF, Li T, Lemon DD, Doherty DH, Curry SR, Aitken JF, Mathews AJ, Johnson KA, Smith RD, Phillips GN, Olson JS. Mechanism of NO-induced oxidation of myoglobin and hemoglobin. Biochemistry. 1996;35:6976–83.
33. Varnado CL, Mollan TL, Birukou I, Smith BJZ, Henderson DP, Olson JS. Development of recombinant hemoglobin-based oxygen carriers. Antioxidants Redox Signaling. 2013;18(17)
34. Laillett DH, Olson JS. In: Winslow RM, editor. Designing recombinant hemoglobin for use a blood substitute. Elsevier; 2006. p. 354–74.
35. Doherty DH, Doyle MP, Curry SR, Valli RJ, Fattor TJ, Olson JS, Lemon DD. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. Nat Biotechnol. 1998;16:672–6.
36. Tsai AG, Kerger H, Intaglietta M. In: Winslow RM, Vandegriff KD, Intaglietta M, editors. Blood substitutes: physiological basis of efficacy. Boston: BirkHauser; 1995. p. 155–74.
37. Burhop K, Gordon D, Estep T. Review of hemoglobin -induced myocardial lesions. Artif Cells Blood Substit Immobil Biotechnol. 2004;32(3):353–74.
38. Hutter J, Hermann J, Juri G, Messmer K. Moderate hemodilution with rHb2.0 for injection. In: Messmer K, Burhop KE, Hutter J, editors. Microcirculatory effects of hemoglobin solutions, vol. 25. Prog Appl Microcirc Karger; 2004. p. 65–74.
39. Hermann J, Hutter J, Messmer K. rHb2.0 for injection for resuscitation from hemorrhagic shock. In: Messmer K, Burhop KE, Hutter J, editors. Microcirculatory effects of hemoglobin solutions, vol. 25. Prog Appl Microcirc Karger; 2004. p. 75–85.
40. von Dobschuetz E, Hutter J, Hoffmann T, Messmer K. Influence of hemoglobin-based oxygen carriers with different nitric oxide scavenging properties on the pancreatic microcirculation after hemorrhagic shock. In: Messmer K, Burhop KE, Hutter J, editors. Microcirculatory effects of hemoglobin solutions. Prog Appl Microcirc Karger; 2004. p. 86–94.
41. Burhop K, Doyle M. The development and preclinical testing of a second generation recombinant hemoglobin solution, rHb2.0 for injection. In: Messmer K, Burhop K, Hutter J, Karger S, editors. Progress in applied microcirculation: 17th Bodensee symposium on microcirculation, vol. 25. AG; 2004. p. 48–64.
42. Young DB, Garbe TR. Lipoprotein antigens of Mycobacterium tuberculosis. Res. Microbiol. 1991;142:55–65.

29

O-Raffinose Cross-Linked Human Hemoglobin (Hemolink): History, Clinical Trials and Lessons Learned

Agya B. A. Prempeh and Davy C. H. Cheng

Introduction

Blood has essential physiologic functions such as tissue oxygenation, immunity provision, hemostasis regulation, and pH buffering. Despite advances in blood conservation strategies and blood management programs, allogenic blood transfusion remains significant [1]. However, allogenic blood transfusion has potential risks, functional limitations, and logistical constraints. These include exposure to blood-borne diseases, incompatibility reactions, immunomodulation, impaired oxygen unloading capacity of stored blood, and decreased availability [2]. Of note, the infectious risks and the periodic shortages of allogenic blood have been a significant impetus to develop "blood substitutes" as an alternative to allogenic blood transfusion or an adjunct to blood conservation strategies [3]. "Blood substitutes" is a misnomer as these agents only serve the oxygen transport and tissue oxygenation function of whole blood. They are more appropriately referred to as oxygen therapeutic agents. These agents are solutions that utilize either hemoglobin or perfluorocarbon as their oxygen carrier [2].

History of Hemoglobin-Based Oxygen Carriers

Research into alternatives for allogenic blood began centuries ago. In 1850, Dr. Hodder injected fresh milk into three moribund patients' veins during the Asiatic cholera pandemic in Canada. Two of the patients recovered from Cholera. About 28 years later, Dr. Thomas of New York was confronted with a moribund patient requiring blood transfusion post gynecological oncology surgery. Recalling Hodder's actions during the pandemic, Dr. Thomas proceeded to do something similar. He administered fresh cow milk into the patient's median basilic vein as he was reluctant to give whole blood due to the coagulation difficulties associated with blood transfusion at the time. The patient steadily improved, and within 6 weeks, she had fully recovered and returned home. Two other moribund patients in his subsequent studies with lacteal infusions, however, did not survive. Dr. Thomas postulated that intravenous infusion of fresh cow's milk was therapeutically beneficial in improving the quantity and quality of blood in humans. He pointed out that lymphatic chyle and milk shared similar chemical properties and suggested that chyle is the substance from which blood is formed by nature. Dr. Thomas was of the view that the deaths of the two patients were unrelated to the lacteal infusions [4].

As a result of Thomas's mixed study findings, oxygen therapeutics' production pivoted to hemoglobin-based solutions in the early twentieth century. Amberson and colleagues conducted animal studies with hemoglobin in lactated Ringer's solution. In a stepwise approach, they exsanguinated cats and replaced the blood loss with hemoglobin solutions. Although there was significant renal dysfunction in the cats due to the therapy, the hemoglobin solution had demonstrated that it could sustain life. The cats survived on a short-term basis, they were able to walk, and their neurological functions were confirmed intact [5]. Amberson's team proceeded to conduct clinical trials using a hemoglobin-containing product in lactated Ringer's solution. Their study was abandoned when 5 out of 14 patients developed significant renal injury and vascular hypertension [6]. In the 1950s, 47 anemic mariners of the US Navy were treated with hemoglobin solutions. Some of the mariners who were treated experienced hypertension and renal toxicity. It was postulated that the hemoglobin and the red cell

A. B. A. Prempeh
Department of Anesthesia & Perioperative Medicine, London Health Sciences Centre, Western University, London, ON, Canada
e-mail: Agya.Prempeh@lhsc.on.ca

D. C. H. Cheng (✉)
Department of Anesthesia & Perioperative Medicine, Schulich School of Medicine, Western Ontario University, London, ON, Canada

School of Medicine, The Chinese University of Hong Kong, Shenzhen, China
e-mail: davy.cheng@uwo.ca

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_29

stroma deposits obstructed the renal tubules, and the hemoglobin moiety induced vasoconstriction, thereby decreasing renal blood flow [7]. The growing evidence of renal and vascular complications associated with hemoglobin-based solutions led to a decline in production. In the 1980s, interest in these agents re-kindled mainly due to the HIV pandemic, hepatitis C transmission, enhanced understanding of their pharmacology, and the evolution of ultra-purification methods. These developments were the catalysts for the production of numerous modified stroma-free hemoglobin-based solutions to improve the hemoglobin performance and decrease its side effects [8].

Four categories of HBOCs have subsequently been produced, namely cross-linked, polymerized, conjugated, and encapsulated. The prototype of the cross-linked group was diaspirin cross-linked hemoglobin (HemAssist). HemAssist consisted of intramolecularly cross-linked alpha sub-units of human hemoglobin. Although the intramolecular covalent bond seemed to stabilize HemAssist and thereby reduce renal toxicity, this product caused intense vasoconstriction with a reduction in cardiac output. Clinical trials on HemAssist were terminated due to increased mortality rates of participants [9, 10]. Polymerized hemoglobin emerged as a viable solution to the vasoconstriction effects of HBOCs. This group of hemoglobin-based solutions was made up of intermolecular cross-linked hemoglobin molecules to increase their molecular size. It was postulated that the increase in these compounds' molecular size would minimize their extravasation from the circulation and subsequent binding to subendothelial nitric oxide. The net result would be the maintenance of the physiologic vasodilatory function of nitric oxide on smooth muscle cells [11]. The first generation of polymerized HBOCs included Hemopure, Oxyglobin, PolyHeme, and Hemolink. Hemopure and Oxyglobin are both bovine-derived HBOC. Hemopure was approved in South Africa to treat anemia in adult surgical patients and Russia to manage acute anemia, irrespective of the underlying etiology. There were safety concerns about Hemopure increasing the risk of strokes and myocardial infarction in some clinical studies; hence it has not yet been approved in the US. However, the US Food and Drug Administration (FDA) has made Hemopure available to patients with severe and life-threatening anemia for whom blood transfusion is not an option and who have exhausted all other management options. Oxyglobin is the only HBOC approved by the US FDA and the European Medicines Agency (EMA), but it is restricted to veterinary use. PolyHeme was a human-derived pyridoxylated polymerized hemoglobin. Clinical trials showed that PolyHeme was neither superior nor inferior to the standard of care management utilized [9]. Hemolink was a unique human-derived HBOC. It was a hybrid of the cross-linked and the polymerized groups of HBOCs, with intramolecular and intermolecular bonds. This product made it to Phase III clinical trials but was not approved for marketing. A new generation of conjugated and encapsulated HBOCs is currently in clinical trials [9].

Properties of Hemoglobin-Based Oxygen Carriers

Hemoglobin extracted from the red cell (cell-free hemoglobin) to form a solution loses many properties to transport oxygen and maintain tissue oxygenation efficiently [8]. Thus, the following properties must be carefully considered to prepare these solutions to ensure safety and efficacy.

Deficiency of Red Blood Cell Membrane

The hemoglobin solutions are relatively blood antigen-free; hence they generally do not need to be cross-matched. However, there are concerns about the development of antibodies to xenogenic hemoglobin [12].

Deficiency of Red Blood Cell

2,3-Diphosphoglycerate (2,3-DPG) is produced by the red blood cell. It binds with greater affinity to the deoxygenated hemoglobin beta sub-units and decreases hemoglobin's affinity for oxygen, thus promoting oxygen unloading at the cellular level. The absence of 2,3-DPG leads to a leftward shift of the oxygen-hemoglobin dissociation curve with resultant inefficiency of oxygen unloading. Early modifications, made to the human-derived hemoglobin, shifted the oxygen-hemoglobin dissociation curve closer to $P_{50} = 26$ mmHg at pH = 7.40, with the assumption of improving oxygen unloading [11]. Bovine hemoglobin is chloride-dependent and does not depend on 2,3-DPG. Hence bovine hemoglobin's affinity for oxygen in humans is within the physiological range because of the chloride ions in human plasma [13].

Source of Hemoglobin Molecule

The principal sources are human, bovine, recombinant, and transgenic technologies. There is no evidence of one source being statistically better in function. Human hemoglobin

tends to be most readily available. Bovine supply is relatively unlimited [11].

Structure of Hemoglobin Molecule

Early theories stated that the unmodified tetrameric form of hemoglobin was responsible for the renal and vascular complications seen in Hemoglobin-Based Oxygen Carriers. The unmodified tetrameric form of hemoglobin, free of the red cell membrane, dissociated in human plasma into dimers and monomers. The renal tubules rapidly filtered them with resultant shortened intravascular half-lives (1–2 hours) and tubular obstruction. The remaining undissociated tetrameric forms in plasma extravasated to bind subendothelial nitric oxide, resulting in unopposed smooth muscle contraction.

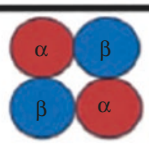
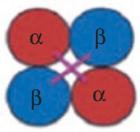
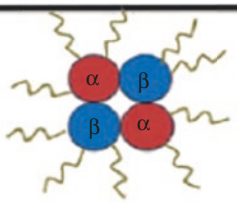
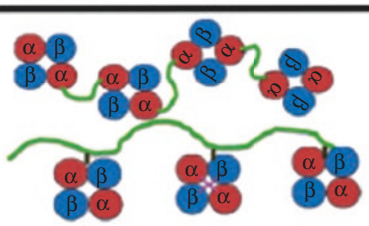
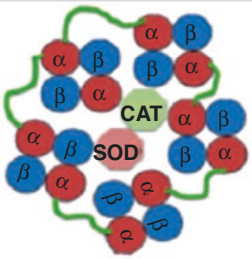
Chemically modified Hb based HBOCs	Materials used	Representative Design Names
 Pure cell-free Hb	Cell-free Hb obtained from human, bovine, salmon and recombinant sources	Cell-free Hb
 Intramolecularly crosslinked Hb	Cell-free Hb crosslinked between subunits, with agents like glycine, glutaraldehyde, O-raffinose, 3,5-dibromosalicyl fumarate, pyridoxal-5-phospate etc.	HemAssist (Baxter) Hemopure (BioPure) Optro (Somatogen) Hemolink (Hemosol) etc.
 PEG-conjugated Hb	Cell-free Hb conjugated on the surface by Maleimide-activated Polyethylene glycol (i.e. Hb-Mal-PEG)	Hemospan (Sangart) etc.
 Intermolecularly crosslinked and polymer-tethered Hb	Cell-free Hb intra-and inter-molecularly crosslinked or tethered from polymer chains by agents like glutaraldehyde, poly-oxy ethylene, O-raffinose etc.	PolyHeme (Northfield Lab) Pyridoxylated Hb or PHP (Apex Bioscience) etc.
 Polymerized Hb with RBC-relevant redox enzymes	Cell-free inter-molecularly crosslinked or polymer-tethered in multiple copies and associated with enzymes like superoxide dismutase (SOD), catalase (CAT) etc that can maintain redox environment for efficient Hb activity	Poly-Hb-SOD-CAT etc.

Fig. 29.1 Representative approaches and design schematics for HBOCs based on the chemical modification [12]

The tetrameric configuration of hemoglobin became a target for modification to ensure stability and increase in molecular weight [11] (See Fig. 29.1).

Iron Moiety

Ferrous (Fe^{2+}) iron is the oxygen-carrying iron in hemoglobin. When hemoglobin binds to oxygen, the ferrous iron is oxidized to the ferric state (Fe^{3+}), and the hemoglobin formed is methemoglobin. Methemoglobin is unable to bind oxygen. The ferric iron (Fe^{3+}) in methemoglobin must be reduced to ferrous iron (Fe^{2+}) to maintain the oxygen-carrying capacity of hemoglobin and normal vascular tone regulation. The reducing enzyme in this redox reaction is NAD-cytochrome b5 reductase, found in red blood cells. Thus, minimization of methemoglobin formation is a consideration in developing Hemoglobin-Based Oxygen Carriers [14].

Properties of Hemolink

Hemosol Incorporated in Canada produced Hemolink. It is an o-raffinose cross-linked and oligomerized human hemoglobin. The manufacturing process begins with the washing of approved red blood cells to exclude plasma and other

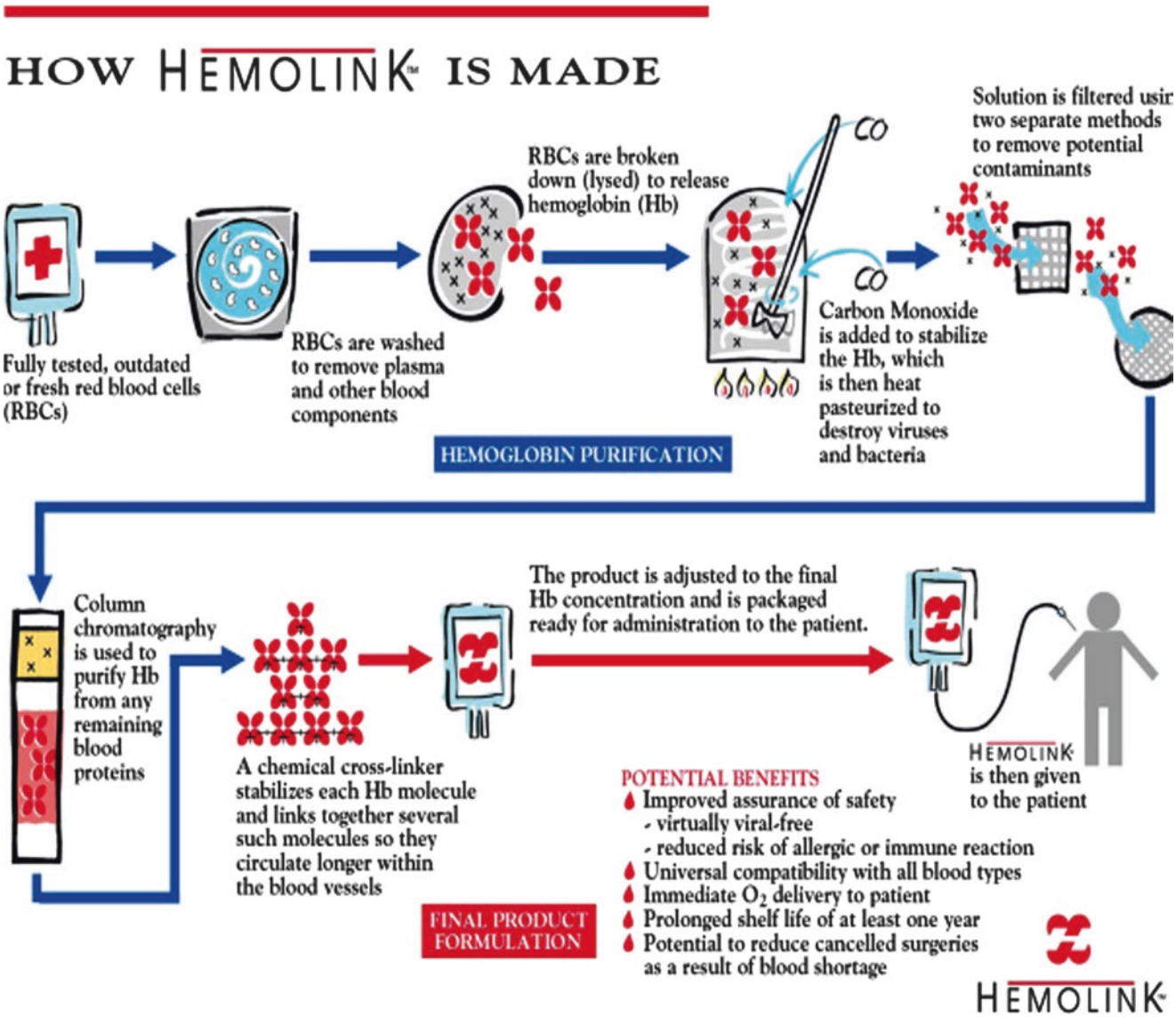


Fig. 29.2 Manufacturing process for o-raffinose cross-linked human hemoglobin [13]

blood cellular components (See Fig. 29.2). The washed red blood cells are lysed to extract hemoglobin A (Hb A). The extracted Hb A is purified through a series of processes, namely pasteurization, filtration, and cation/anion chromatography. The final Hb A purity is greater than 99%. The purified Hb A tetramer forms both intra- and intermolecular bonds to increase its stability and molecular weight. The intramolecular covalent bond of the Hb A tetramer occurs at the two beta sub-units within the 2,3-DPG binding pocket to form a stable 64 kDa molecule. Multiple stabilized Hb A tetramers are cross-linked via o-raffinose polyaldehyde to form stable polymers of 128–600 kDa molecular weight. The final product contains 100 g $Hb{\cdot}L^{-1}$ in a Lactated Ringer's solution. The solution contains >95% intra- and-intermolecular bonds, of which >55% are intermolecular bonds (i.e., polymerized) [13]. Other properties of the solution [15] include kinematic viscosity = 1.24 centistokes, oncotic pressure = 26 mmHg, osmolality = 280–300 mOsm/L, P_{50} = 39 ± 12 mmHg at pH = 7.50 ± 0.5, non-cooperative behavior (Hill coefficient (n_{50}) = 1.0 ± 0.2), and the endotoxin levels <0.06 EU/ml. Hemolink has a half-life = 14–20 hours [16].

Clinical Trials of Hemolink

The development of Hemolink commenced with discovery research followed by pre-clinical studies. Based on the pre-clinical studies' data, applications were made to regulatory authorities to conduct clinical trials. Hemosol Incorporated completed eight clinical trials on Hemolink (four in cardiovascular surgery, two in orthopedic surgery; one in anemic patients; and one in healthy volunteers). The key highlights being the clinical trials for primary CABG surgery, which included a Phase III clinical trial in Canada and the United Kingdom.

There are two types of study designs used in cardiac surgery to evaluate oxygen therapeutic agents [17]. The primary outcome of both designs is usually avoiding transfusion of allogenic blood. In the first study design, all participants undergo intraoperative autologous donation (IAD). The harvested blood is replaced with an equivalent volume of either the investigational product or a colloid solution in a randomized manner. This design's perceived advantage is that some control arm participants may not require an allogenic blood transfusion. In the alternate study design, the participants do not undergo IAD. A universal blood transfusion threshold is pre-determined for all the participants. The participants are randomized to receive either the investigational product or allogenic blood when the threshold is reached. This design's drawback is that all the control arm participants that reach the threshold receive allogenic blood. Researchers tend to use the first study design involving IAD to evaluate oxygen therapeutics in cardiac surgery, while the alternate study design is mainly used in non-cardiac surgical studies.

Phase I: Safety Trial

The study was randomized, placebo-controlled, and double-blinded. The objective of the study was to assess the safety of Hemolink in healthy volunteers. Forty-two healthy adult male volunteers between the ages of 19–39 years were enrolled. The participants received either an intravenous Hemolink 10% (w/v) solution or an equivalent volume of lactated Ringer's solution. The doses of Hemolink administered ranged from 0.025 to 0.6 gHb/kg, i.e., 1.7–42 g. The participants were monitored for 3 days in the clinical facility with a 6-week follow-up. There was a dose-dependent increase in mean arterial pressure with a plateau of approximately 14% above baseline at 0.1 gHb/kg, with an associated decrease in heart rate without electrocardiographic abnormalities. Respiratory function remained unchanged. Some participants experienced abdominal pain of moderate-to-severe intensity, which was alleviated with antispasmodics. The pain was observed at doses >0.4 gHb/kg. There was also a dose-dependent increase in serum bilirubin with values above the upper limit of normal at doses >0.4 gHb/kg. Two participants had elevated creatine kinase concentrations but with normal creatine kinase-MB mass fractions. The hematologic markers remained within the normal limits [18].

Phase II: CABG Trial

A prospective randomized, controlled, and open-label multi-center trial was conducted in Canada and the United Kingdom. The study aimed to determine the dose-response of Hemolink administered as an adjunct to IAD in patients undergoing coronary artery bypass grafting surgery (CABG). A secondary objective was to assess the efficacy of Hemolink in reducing the incidence of allogenic blood transfusion. Sixty male and female participants between the ages of 18–75 years were enrolled in the study. The participants must have had a hematocrit adequate to allow pre-bypass harvesting of 500–1500 ml of autologous blood to yield a hematocrit of 0.20–0.21 while on cardiopulmonary

bypass. Exclusion criteria included congestive heart failure, reduced left ventricular ejection fraction (<30%), recent myocardial infarction (less than 4 weeks), unstable angina requiring the use of heparin and/or iv nitroglycerin, a history of a cerebral vascular accident including transient ischemic attack, or any significant co-morbidity outside the cardiovascular system. After the pre-bypass harvest of 500–1500 ml of autologous blood, 30 participants received a single dose of Hemolink in an escalation sequence (250, 500, 750, 1000 ml) while the remaining 30 participants in the control arm received an equivalent volume of 10% Pentastarch. A pre-defined transfusion threshold was set for blood (autologous/allogenic) to be administered. Seventeen percent of the control arm participants required intraoperative allogenic blood transfusion compared with zero in the Hemolink group ($p = 0.052$). This apparent advantage of Hemolink was maintained at 24 hours after surgery (7% vs. 37%; $p = 0.010$) and up to 5 days after surgery (10% vs. 47%; $p = 0.0034$). All serious adverse events were considered unrelated or unlikely to be related to the investigatory product. Most adverse events (98%) were mild or moderate in severity [19].

Phase III: CABG Trial

This trial was a prospective, randomized, controlled, double-blinded study conducted in 24 centers in Canada and the United Kingdom. The study's primary purpose was to evaluate the efficacy of 750 ml Hemolink, as an adjunct to IAD, in decreasing the incidence of allogenic blood transfusion during and after primary CABG surgery. All participants underwent IAD. The harvested blood was replaced with either 750 ml Hemolink or 750 ml Pentastarch. The participants were transfused with blood when a predetermined transfusion threshold was reached. IAD blood first, then allogenic blood if indicated. The participants were followed up for 3–8 weeks upon hospital discharge. Although 386 participants were enrolled in the study, only 299 participants received Hemolink or Pentastarch. Eleven of the 299 participants experienced unusual intraoperative events and blood loss. The researchers decided the unusual events were unlikely to be related to the use of either Hemolink or Pentastarch. The remaining 288 participants were designated as the "unplanned efficacy analysis" (UEA) population. The efficacy analysis results showed that 27% of the control group participants received allogenic blood compared with 17% of the Hemolink group participants.

Furthermore, the Hemolink group required lesser quantities of allogenic blood or blood products. There was a delay in the time to transfusion of allogenic blood in the Hemolink group. Upon completing the Phase III trial in the second quarter of the Year 2000, Hemosol filed a marketing authorization application with the UK Medicines Control Agency and Health Canada, but approval was declined. The regulatory authorities requested additional studies to supplement the information in the clinical experience section. The Phase III trial results are yet to be published in a peer-reviewed journal [15, 20].

Discontinuation of Clinical Trials

Hemosol Incorporated commenced the US Phase III CABG Trial in the fourth quarter of the Year 2000. The FDA later requested an amendment to the clinical trial protocol for Hemolink to strengthen the efficacy analysis. As a result of the discussions with the FDA, Hemosol Incorporated suspended their ongoing Phase III trial to develop a more comprehensive Phase II CABG trial in the US, which would provide grounding for the resumption of the Phase III clinical trial protocol for Hemolink. The FDA subsequently approved four other Phase II clinical trials for Hemolink. The trials included Hemolink in re-do CABG surgery, chemotherapy-induced anemia, patients undergoing high blood loss orthopedic surgery, and patients with severe acute anemia for whom red cell transfusion was not a therapeutic option. In March 2003, the Data and Safety Monitoring Board (DSMB) of the Phase II-Primary CABG trial in the US notified Hemosol Incorporated that it had observed an imbalance in the incidence of cardiac adverse events in the Hemolink group. Hemosol Incorporated elected to suspend all Phase II clinical trials involving the use of Hemolink pending an internal and external safety analysis audit. Based on the audit reports, Hemosol Incorporated concluded that further pre-clinical studies of Hemolink were imperative before re-commencing the clinical trials [20].

Lessons Learned

Despite substantial sponsored-research investments by the National Institutes of Health, industries, and militaries in the 1980s and 1990s to develop oxygen therapeutic agents, none of the products are currently approved for clinical use in North America or Europe [21]. However, the vast amount of research on these agents has added to the body of knowledge in this field [21].

Although decades of modern research have shown differences in the oxygen transport and tissue oxygenation properties between oxygen therapeutic agents and allogenic blood,

clinical comparison of these two compounds has potential challenges. The adverse events related to allogenic blood transfusion happen infrequently. Hence a large population of participants would be required to adequately power a clinical study to compare the safety profiles of oxygen therapeutic agents and allogenic blood. An option to solve this conundrum would be to demonstrate an acceptable safety profile and clinical equivalence of oxygen therapeutic agents to blood in settings where allogenic blood may not be readily available. An alternative approach would be to utilize the unique oxygen delivery properties of these agents. The goal would be to provide temporary regional oxygenation in hypoxic or ischemic tissue [21]. Animal experiments by Fontes showed that machine perfusion with HBOC enriched perfusate appeared to be efficacious in improving the condition of prolonged cold ischemic liver grafts before transplantation [22]. Subsequently, the first human liver transplantation after machine perfusion with HBOC has been performed [23].

At the macro-circulation, viscosity is an essential determinant of the ability of HBOCs to transport oxygen to the tissues. Hemoglobin solutions are much less viscous than whole blood. Thus, hemodilution with HBOCs solutions lowers whole blood viscosity and systemic vascular resistance. The reduced blood viscosity would potentially improve the macro-circulatory flow [24]. The microcirculation, on the other hand, is complex. Flow in this circuit is regulated by many factors, including shear stress and the local oxygen partial pressure.

The endothelial cells lining the small vessels in the microcirculation sense shear stress. Shear stress is directly proportional to viscosity and colloid oncotic pressure [25]. A reduction in the shear stress in the microcirculation triggers endothelial cells to down-regulate nitric oxide production [26, 27]. Theoretically, HBOCs with high colloid oncotic pressure would be expected to maintain flow in the microcirculation. The assumption is that the hemodilution effect of HBOCs would balance the high colloid osmotic press [28, 29]. When HBOCs are normalized for hemoglobin concentration, the polymerized hemoglobin tetramers have lower colloid oncotic pressure than those consisting of stabilized hemoglobin tetramers or surface conjugates. Thus, polymerized hemoglobin tetramers may have reduced efficacy in maintaining microcirculation flow when only shear stress is considered.

Researchers have established that terminal arterioles regulate the microcirculation flow in response to the partial pressure of oxygen to match the tissue's oxygen demands [30]. Paradoxically, increased oxygen delivery to the terminal arterioles triggers vasoconstriction [29–31]. Factors that determine oxygen delivery to the [31–33] terminal arterioles include the blood's oxygen content, hemoglobin ability to unload oxygen, and oxygen's ability to diffuse from the hemoglobin molecule to the vascular endothelium. The diffusion coefficient of a molecule is inversely related to its molecular radius [32–34]. Accordingly, HBOCs with smaller molecular radii, and presumably higher diffusion coefficients for oxygen, have been shown to produce vasoconstriction and limit blood flow to distal capillary beds [34–36]. Experimental models suggest that the diffusion property of an HBOC is a significant contributor to its vasoactivity compared with its oxygen affinity, P50 [34–36]. These studies suggest that an HBOC with high viscosity, high colloid osmotic pressure, and large molecular radius is less likely to trigger a vasoconstrictive response, thus improving flow and oxygen delivery to the capillary beds.

The modern safety concerns of HBOCs have been mainly attributed to their vasoactivity and systemic pressor effect [37]. The vasoactive and systemic pressor effects were mainly attributed to the nitric oxide scavenging theory in earlier times. It was postulated that HBOCs with large molecular weights would not readily extravasate into the subendothelial space to bind to nitric oxide to cause unopposed vasoconstriction. Subsequently, this hypothesis was challenged when significant vasoconstriction effects were observed in PolyHeme and Hemopure, composed almost entirely of higher-order polymers of hemoglobin with minimal residual tetramer [38].

In conclusion, the profound insight into the pharmacological properties of oxygen therapeutic agents, made possible related to previous generations' failure, has been a silver lining. Although past failure modulates optimism, the more recent agents are promising as they are specifically designed to supply ischemic or hypoxic tissue with oxygen [21]. It is still too early to determine if these newer-generational products will succeed. However, the scientific advances made in their development will undoubtedly advance the field of oxygen therapeutics towards ultimate success in blood substitutes, to the benefit of the population.

Key Points

- The use of allogenic blood is common worldwide; its associated risks and periodic shortages have spurred the development of "substitutes", particularly hemoglobin-based oxygen carriers (HBOCs).
- Hemolink was a unique hybrid HBOC. It was composed of stabilized tetrameric human hemoglobin A units, which were cross-linked with o-raffinose polyaldehyde to form polymers.
- Despite Hemolink's observed potential in clinical trials in the UK and Canada, its adverse vasopressor effects became more apparent with increased cardiac events in the US clinical trials.
- Emerging theories on the pathophysiology of the vascular complication of HBOCs suggest the microcirculation dynamics play a more important role than the nitric oxide scavenging hypothesis.

- There is cautious optimism that the new generation of HBOCs will succeed, given the vast body of knowledge accumulated on their pharmacological properties.

References

1. Meier J, Filipescu D, Kozek-Langenecker S, Llau Pitarch J, Mallett S, Martus P, et al. Intraoperative transfusion practices in Europe. Br J Anaesth. 2016;116(2):255–61.
2. Chen JY, Scerbo M, Kramer G. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. Clinics (Sao Paulo). 2009;64(8):803–13.
3. Stowell CP. What happened to blood substitutes? Transfus Clin Biol. 2005;12(5):374–9.
4. Thomas TG. The intra-venous injection of milk as a substitute for the transfusion of blood: illustrated by seven operations. N Y Med J. 1878;27: p. 449.
5. Amberson WR, Mulder AG, Steggerda FR, Flexner J, Pankratz DS. Mammalian life without red blood corpuscles. Science. 1933;2014(78):106–7.
6. Amberson WR, Jennings JJ, Rhode CM. Clinical experience with hemoglobin-saline solutions. J Appl Physiol. 1949;1(7):469–89.
7. Winslow RM. The results of 62 large-volume hemoglobin infusions in man. In: Hemoglobin-based red cell substitutes. Baltimore and London, Johns Hopkins University Press; 1992. p. 177–8.
8. Carmichael FJ. Recent developments in hemoglobin-based oxygen carriers – an update on clinical trials. Transfus Apher Sci. 2001;24(1):17–21.
9. Moradi S, Jahanian-Najafabadi A, Roudkenar MH. Artificial blood substitutes: first steps on the long route to clinical utility. Clin Med Insights Blood Disord. 2016;9:33–41.
10. Shekhar C. Blood relatives: artificial oxygen carriers between promise and concern. Chem Biol. 2007;14(10):1091–2.
11. Gould SA, Moss GS. Clinical development of human polymerized hemoglobin as a blood substitute. World J Surg. 1996;20(9):1200–7.
12. Jahr JS, Walker V, Manoochehri K. Blood substitutes as pharmacotherapies in clinical practice. Curr Opin Anaesthesiol. 2007;20(4):325–30.
13. Anbari KK, Garino JP, Mackenzie CF. Hemoglobin substitutes. Eur Spine J. 2004;13 Suppl 1(Suppl 1):S76–82.
14. Gupta AS. Hemoglobin-based oxygen carriers: current state-of-the-art and novel molecules. Shock. 2019;52(1S Suppl 1):70–83.
15. Cheng DC. Safety and efficacy of o-raffinose cross-linked human hemoglobin (Hemolink) in cardiac surgery. Can J Anaesth. 2001;48(4 Suppl):S41–8.
16. Scatena R, Giardina B. O-raffinose-polymerised haemoglobin. A biochemical and pharmacological profile of an oxygen carrier. Expert Opin Biol Ther. 2001;1(1):121–7.
17. Mazer D. Review of clinical trials of oxygen therapeutics. TATM. 2000; SI, 27–31.
18. Carmichael FJ, Ali AC, Campbell JA, Langlois SF, Biro GP, Willan AR, et al. A phase I study of oxidized raffinose cross-linked human hemoglobin. Crit Care Med. 2000;28(7):2283–92.
19. Cheng DC, Mazer CD, Martineau R, Ralph-Edwards A, Karski J, Robblee J, et al. A phase II dose-response study of hemoglobin raffimer (Hemolink) in elective coronary artery bypass surgery. J Thorac Cardiovasc Surg. 2004;127(1):79–86.
20. Securities and Exchange Commission, Washington, DC 20549. Form 6-K. Report of foreign issuer pursuant to rule 13a-16 or 15d-16 of The Securities Exchange Act of 1934. Hemosol Corp 2004.
21. Winslow RM. Current status of oxygen carriers ('blood substitutes'): 2006. Vox Sang. 2006;91(2):102–10.
22. Fontes PA. The evolution of oxygen carrier solutions for machine perfusion. Transplantation. 2017;101(11):2657–8.
23. Spahn DR, Goodnough LT. Alternatives to blood transfusion. Lancet. 2013;381(9880):1855–65.
24. Winslow RM, Gonzales A, Gonzales ML, Magde M, McCarthy M, Rohlfs RJ, et al. Vascular resistance and the efficacy of red cell substitutes in a rat hemorrhage model. J Appl Physiol. 1998;85:993–1003.
25. Karmakar N, Dhar P. Effect of steady shear stress on fluid filtration through the rabbit arterial wall in the presence of macromolecules. Clin Exp Pharmacol Physiol. 1996;23(4):299–304.
26. de Wit C, Schäfer C, von Bismarck P, Bolz SS, Pohl U. Elevation of plasma viscosity induces sustained NO-mediated dilation in the hamster cremaster microcirculation in vivo. Pflugers Arch. 1997;434(4):354–61.
27. Intaglietta M, Johnson PC, Winslow RM. Microvascular and tissue oxygen distribution. Cardiovasc Res. 1996;32(4):632–43.
28. Fischer SR, Burnet M, Traber DL, Prough DS, Kramer GC. Plasma volume expansion with solutions of hemoglobin, albumin, and ringer lactate in sheep. Am J Phys. 1999;276(6):H2194–203.
29. Migita R, Gonzales A, Gonzales ML, Vandegriff KD, Winslow RM. Blood volume and cardiac index in rats after exchange transfusion with hemoglobin-based oxygen carriers. J Appl Physiol (1985). 1997;82(6):1995–2002.
30. Duling BR, Berne RM. Longitudinal gradients in periarteriolar oxygen tension. A possible mechanism for the participation of oxygen in local regulation of blood flow. Circ Res. 1970;27(5):669–78.
31. Tsai AG, Vandegriff KD, Intaglietta M, Winslow RM. Targeted O2 delivery by low-P50 hemoglobin: a new basis for O2 therapeutics. Am J Phys Heart Circ Phys. 2003;285(4):H1411–H9.
32. Wettstein R, Tsai AG, Erni D, Winslow RM, Intaglietta M. Resuscitation with polyethylene glycol-modified human hemoglobin improves microcirculatory blood flow and tissue oxygenation after hemorrhagic shock in awake hamsters. Crit Care Med. 2003;31(6):1824–30.
33. Cabrales P, Tsai AG, Winslow RM, Intaglietta M. Effects of extreme hemodilution with hemoglobin-based O2 carriers on microvascular pressure. Am J Physiol Heart Circ Physiol. 2005;288(5):H2146–53.
34. McCarthy MR, Vandegriff KD, Winslow RM. The role of facilitated diffusion in oxygen transport by cell-free hemoglobins: implications for the design of hemoglobin-based oxygen carriers. Biophys Chem. 2001;92(1–2):103–17.
35. Vaslef SN, Kaminski BJ, Talarico TL. Oxygen transport dynamics of acellular hemoglobin solutions in an isovolemic hemodilution model in swine. J Trauma. 2001;51(6):1153–60.
36. Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, et al. Molecular dimensions of Hb-based O(2) carriers determine constriction of resistance arteries and hypertension. Am J Physiol Heart Circ Physiol. 2000;279(3):H908–15.
37. Buehler PW, Alayash AI. Toxicities of hemoglobin solutions: in search of in-vitro and in-vivo model systems. Transfusion. 2004;44(10):1516–30.
38. Rohlfs RJ, Bruner E, Chiu A, Gonzales A, Gonzales ML, Magde D, et al. Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. J Biol Chem. 1998;273(20):12128–34.

PolyHeme: History, Clinical Trials, and Lessons Learned

30

Alexis Cralley and Ernest Moore

Product Development of PolyHeme

PolyHeme was the first human polymerized hemoglobin (Hb) product evaluated in severely injured patients, and was intended for use when stored red blood cells (RBCs) would be unavailable. The product was produced by Northfield Laboratories (Evanston, IL) and development began as early as 1969 through collaboration with the United States Army (although Northfield Laboratories was not officially founded until 1985) [1].

Creation of PolyHeme began with the collection of expired human blood. The blood is washed using pyrogen-free water resulting in lysis of the red blood cells. Further filtration results in the separation of the RBC membrane and components (stroma) from the Hb molecules. At this stage the resulting product is stroma free Hb (SFH) [2–4]. Northfield Laboratories chemical modification of SFH involved a 2-step process that began with pyridoxylation, followed by polyermization of the Hb molecules [5]. First, pyridoxal 5-phosphate was added to the SFH solution in a 4:1 molar ratio. The mixture was deoxygenated through nitrogen gas exchange. As oxygen is removed, sodium borohydride is added to the solution and the reaction is allowed to proceed for 2–4 hours resulting in pyridoxalated Hb [4]. The pyridoxylation of SFH product increases the P50 from 12–14 mm Hg to 20–22 mm Hg, offsetting the increased oxygen affinity that occurs following the loss of 2,3-diphosphoglycerate during RBC lysis. Pyridoxylation allows for improved unloading of oxygen at capillary beds compared to SFH. Early pre-clinical models using primates and transfusion exchange showed that Northfield's pyridoxylated Hb successfully resulted in increased venous PO2 and improved unloading of oxygen at higher tissue PO2 levels compared to SFH [6].

The next step, polymerization of the SFH-P molecules prevented dissociation of the tetrameric Hb, and the associated side effects reported in the 1978 clinical trial by Savitsky [7], and also served to raise the Hb concentration while decreasing the colloid oncotic pressure to a physiological tolerable level [6]. Northfield used glutaraldehyde to create intermolecular crosslinking of Hb molecules. Polymerization was allowed to occur until the measured colloid oncotic pressure decreased to the desired physiologic levels. Following polymerization, all unreacted tetramer was removed resulting in a final pure polymerized stroma free pyridoxylated Hb solution (Poly-SFH-P) [5, 8, 9]. Northfield published limited preclinical animal studies prior to embarking on clinical trials. However, they showed that when used in exchange transfusion in primates, PolyHeme successfully delivered oxygen at a variety of hematocrit ranges. Importantly, this was effective at clinically relevant low hematocrit levels, including lethal levels of near zero hematocrit, and did so without evidence of vasoconstriction or renal dysfunction associated with SFH [10, 11]. For their clinical trials Northfield finalized their PolyHeme product to be comparable to the amount of Hb received in 1 unit of pRBC transfusion, the properties of the 500 mL unit of PolyHeme are shown in Table 30.1.

Table 30.1 Characteristics of 1 unit Polyheme

Volume	500 mL
Total Hemoglobin Mass	50 g
[Hb]	10 g/dL
Molecular Weight (>64 kDa)	99%
P_{50}	26–32 mm Hg
Tetramer	≤1.0%
Methemoglobin %	<8
$t_{1/2}$	24 h
Shelf Life at 4 °C	>1.5 year
Shelf Life at 21 °C	6 weeks

A. Cralley (✉) · E. Moore
Ernest E Moore Shock Trauma Center at Denver Health, Denver, CO, USA

Department of Surgery, University of Colorado Denver, Aurora, CO, USA
e-mail: Alexis.Cralley@dhha.org

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_30

Phase I Clinical Trials

The first open-label, dose escalation safety study occurred at a single site in 22 health male volunteers. Subjects received doses ranging from 9.2 to 61.2 g of PolyHeme injections, resulting in plasma Hb levels from 174 mg/dL to 1.211 g/dL. No clinically meaningful changes in hemodynamics or unwanted systemic side effects were detected or attributed to PolyHeme, supporting the tolerable safety profile of the product [12]. Following evaluation in healthy subjects, 10 male and female adult trauma subjects were enrolled at a single center and treated with one unit of PolyHeme instead of one unit of pRBC. The one unit of PolyHeme increased plasma Hb by 1.3 ± 0.5 g/dL. Significant changes in vital signs, hematology, chemistry and urinalysis were not detected and the one unit of PolyHeme was well tolerated [12]. These Phase I clinical trials were completed in 1991 [13].

Phase II Clinical Trials

The first phase II clinical trial to assess the potential therapeutic benefits of PolyHeme in patients suffering acute blood loss was conducted at Denver Health Medical Center [14]. This prospective, nonrandomized, open label trial enrolled 39 adult trauma patients who provided informed consent to receive either 1, 3, or 6u of PolyHeme. The infusion rate of PolyHeme ranged from 1u in 175 minutes to all 6u in 20 minutes. For the 8 subjects who received all 6 units of PolyHeme, their average pre-infusion Hb concentration was 9.7 ± 2.6 g/dL. The addition of 6 of PolyHeme during active blood loss resulted in total Hb levels of 7.5 ± 1.2 g/dL, even though the RBC Hb concentration fell to 2.9 ± 1.2 g/dL, reflecting the balance between active blood loss and Hb administration. In these patients the plasma Hb concentration provided by PolyHeme rose from 0 to 4.8 ± 0.8, supporting PolyHeme's efficacy to serve as a safe pRBC alternative for up to 6u. Over half of the subjects (n = 23, 59%) received PolyHeme only without any additional blood product transfusions, demonstrating PolyHeme's ability to reduce the need for allogenic blood product for patients who require only 1–2 units of blood. Safety assessment, including patient temperature, mean arterial pressure, heart rate, and creatinine clearance, were measured over a 3 day monitoring period and did not significantly change from baseline.

The first randomized, prospective open labeled clinical trial comparing the efficacy and safety of PolyHeme against pRBC occurred at two Level 1 trauma centers, Denver Health Medical Center and at the University of California at San Diego [15]. 44 adult patients who sustained acute blood loss following trauma or operation and had an urgent need for transfusion were enrolled. The control group received allogenic blood immediately while those randomized to the experimental group received up to 6u of PolyHeme initially during the period of hemorrhage. Any additional transfusion requirements in the PolyHeme group were completed with allogenic transfusion as needed. At the end of the infusion period the experimental group had an average RBC Hb concentration of 5.8 ± 0.5 g/dL compared to 10.6 ± 0.3 g/dL in the control group, however the total Hb between the experimental and control groups was not statistically different due to the additional plasma Hb concentration (4.4 ± 0.3 g/dL) provided by the PolyHeme solution. The PolyHeme group required significantly less allogenic blood units through the first 12 and 24 hours as shown in Table 30.2, resulting in a reduction of allogenic blood transfusions by 4u over the 3 days of monitoring. Overall this first randomized trial demonstrated PolyHeme's ability to not only serve as an acceptable short term blood substitute to maintain Hb concentrations, but also supported PolyHeme's ability to reduce allogenic blood utilization, this time in ongoing bleeding patients [15].

The first trial that increased the potential available PolyHeme dose to 20 units was an open label, nonrandomized, multicenter trial completed in 2002 [16]. A total of 171 patients provided informed consent to receive up to 20u of PolyHeme for the treatment of surgical blood loss. The primary outcome was 30 day mortality in patients receiving PolyHeme compared to a single institution historical control group of 300 consecutive surgical patients. The historical control group consisted of patients who declined blood transfusion for religious preference and developed anemia and Hb <8 during surgery. Mortality increased as Hb decreased for both patients cohorts, and at Hb levels ≤5.6 g/dL mortality in the PolyHeme group was significantly reduced compared to the controls (Fig. 30.1). Furthermore, in a subgroup of patients who had progressive, rapid blood loss resulting in RBC Hb concentrations below 3 g/dL, the PolyHeme transfusions resulted in acceptable total Hb concentration >7 g/dL. The mortality rate for the PolyHeme subgroup was 25% (10/40), compared to 64.5% (20/31) in the historical control group. Furthermore, at RBC Hb levels at 2 g/dL or less there were no survivors in the historical control group, while the PolyHeme group had a survival rate of 75% (9/12) in patients whose RBC [Hb] dropped to <1 g/dL. This trial demonstrated PolyHeme's ability to prevent life-threatening anemia and improve survival during massive hemorrhage when allogenic blood products are unavailable. Northfield attempted to receive FDA clearance following the results of

Table 30.2 Red blood cell units required

Time	Control	Experiment	p-value
Infusion	4.9 ± 1.4	0	$p < 0.05$
1st 12 hours	9.5 ± 4.2	5.2 ± 4.4	$p < 0.05$
1st 24 hours	10.4 ± 4.2	6.8 ± 3.9	$p < 0.05$
Through Day 3	11.3 ± 4.1	7.8 ± 4.2	$p = 0.06$

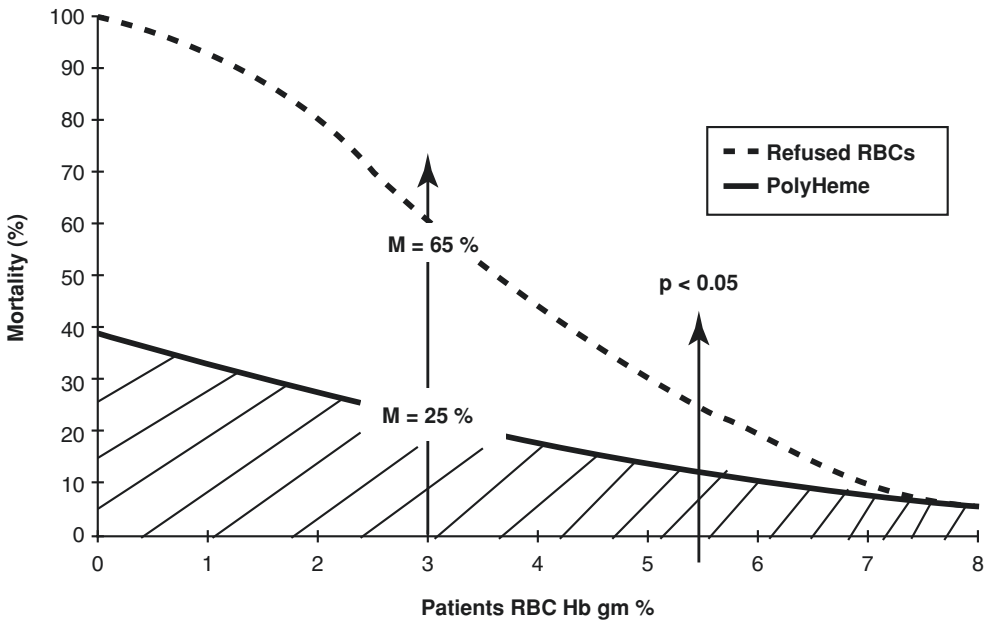


Fig. 30.1 30-Day mortality of Polyheme vs Historical controls. Mortality was significantly less in the Polyheme group when the RBC [Hb] ≤5.6 g/dL. (From: Gould, et al. [16]; with permission)

this trial, however, the FDA returned the application, citing that the product had not yet been tested in ambulance and prehospital settings where it would likely be employed [17].

PolyHeme's Immunoinflammatory Effect Compared to Stored RBCs

Throughout these early clinical trials the Denver Health Medical Center investigator group led by Dr. Ernest Moore also focused on evaluating PolyHeme's use as an RBC product alternative to decrease the rate of multiple organ failure (MOF) in acutely injured trauma patients. The DHMC group was particularly interested in the pro-inflammatory effects of stored RBCs and their capacity to induce MOF through neutrophil cytotoxicity following transfusion [18]. They had previously identified that stored RBCs contain inflammatory cytokines and lipids resulting in high levels of polymorphonuclear leukocytes (PMNs) primed to release cytotoxic products. They found that 6 or more units of pRBCs transfusions during resuscitation was an independent risk factor for the development of MOF [19]. During their PolyHeme clinical trials they conducted in vivo and vitro studies comparing the effects of stored RBCs vs PolyHeme product on isolated PMNs and vascular endothelium. In vitro findings showed that stored RBCs resulted in endothelium activation and leukocyte superoxide production and elastase release while PolyHeme did not produce these proinflammatory effects [20]. In their PolyHeme clinical trials they measured cytotoxic priming during the first 120 hours after infusions of pRBCs or PolyHeme. They found that the levels of PMNs primed for cytotoxicity in the group that received pRBCs was twice as high as the PolyHeme cohort [21]. Additionally, patients who received pRBCs had significantly higher levels of proinflammatory cytokines [22]. They found that of 20 subjects who received PolyHeme for initial resuscitation, only 15% developed MOF compared to a predicted incidence of 37% in this acutely injured population [20]. Their identification of the pro-inflammatory side effects of stored RBCs and the increased associated with MOF after 6 units of stored pRBC transfusions were a key stimulus in formulating the Phase III clinical trial design.

Phase III Clinical Trials

The final, phase III, multicenter randomized, controlled open-label trial initiative begin in 2003. The study employed an exception of informed consent waiver which was granted under FDA regulation 21CFR§50.24. Enrollment occurred from January 2004 through July 2006 at 29 level 1 trauma centers, reaching a total enrollment of 720 patients [23]. Adult subjects who met the inclusion criteria of acute blood loss from trauma with class III hemorrhagic shock were enrolled and randomized in the field. Control patients received standard of care (crystalloid in the field and allogenic blood products as needed at the level one trauma center). The experimental cohort received PolyHeme in the field could receive up to 6u of PolyHeme during the first 12 hours postinjury at the level 1 trauma center. Allogenic blood was transfused as needed after the sixth unit of PolyHeme or if the 12 hour treatment window had passed. This important 12 hour window allowed for sufficient time to transfuse PolyHeme product in patients with short transport times, and also allowed for the secondary evaluation of patients who met massive transfusion requirements of

needing 6 or more units of a Hb carrying solution and were thus at high risk for MOF.

The study was powered for a superiority and noninferiority assessment of Day 30 mortality. The superiority hypothesis stated that PolyHeme patients would have a 7% lower mortality compared to the control patients, and the noninferiority hypothesis stated that PolyHeme patients would have no more than 7% higher mortality than the control group. Secondary endpoints included day 1 mortality rate, mortality comparison of penetrating vs blunt injuries, total allogenic blood use, and the incidence of multiple organ failure by day 30. Overall, 590 patients were treated per protocol. Day 30 mortality was not significantly different between the groups ($p = .196$): 13% in the PolyHeme group (46/349) compared to 10% in the control group (36/365). Day 1 mortality was also not significantly different between the two groups; however, in the per protocol analysis the median time to the first blood transfusion was 14.1 hours in the PolyHeme group compared to 1.5 hours in the control group [23]. The delay in blood product transfusion without differences in mortality rate reflects PolyHeme's clinical ability to prolong survival in setting of delayed RBC transfusion. Additionally, exposure to stored blood product was significantly reduced in the PolyHeme group, with only 43% of the experimental group subjects requiring pRBCs compared to 52% in the control group ($p < 0.001$). Since the overall study enrolled a mix of trauma patients and was not specific to severely injured patients (only half of the control subjects required blood transfusions, and only 34% had massive transfusion requirements of 6 or more units), the incidence of MOF was overall low and not significant between the two groups. Of those patients who developed MOF all but five received at least 6u of pRBCs (18/20 Control, 23/26 PolyHeme).

Another adverse event of particular importance for this study was the rate of myocardial infarctions (MI). In 2008, a JAMA meta-analysis reported an increased risk of MIs associated with all Hb blood substitutes, including PolyHeme [24]. For this clinical trial the initial investigator reported MIs were higher in the PolyHeme group (11 vs 3, $p < 0.05$) and none were considered "possibly" or "probably" related to PolyHeme administration. A blinded, independent Cardiac Event Subcommittee performed a post hoc analysis to adjudicate the rates of cardiovascular adverse events in the study. The subcommittee's independent review identifying "possible" and "probable" MIs resulted in greater than 50% of patients in each group having some evidence of MI. Ultimately their review could not find an association of adverse cardiovascular events (elevated cardiac markers, abnormal EKGs, and MIs) to the use of PolyHeme [23, 25].

Following the results of the Phase III trial Northfield Laboratories submitted for FDA approval of PolyHeme. However, for the noninferiority analysis only the per protocol analysis (n = 590) had the confidence interval range below the prespecified 7% cutoff, while the per treated analysis (n = 714) the confidence interval exceeded the 7% cutoff. In the per protocol analysis, 124 subjects were removed from the analysis due to eligibility violations or treatment protocol violations. Unfortunately, in May 2009 the FDA refused to approve PolyHeme for clinical use citing the inability to meet the predetermined cutoff in the Per Treatment analysis [17].

Ethical Issues and Lessons Learned

Northfield's final PolyHeme clinical trial left two lasting controversial questions in the trauma research field: first, when and how should Waiver of Informed Consents be used for trauma studies? and second, what should the ideal primary endpoint be for evaluating novel trauma interventions in heterogenous populations?

In regards to the first topic of using an informed consent process for trauma research, the PolyHeme Phase III Clinical Trial drew national scrutiny. The Exception for the requirement of informed consent (21 CFR 50.24) was released in 1996 and required several key requirements, all of which the trial met including rigorous community consultation [26]. However, controversy regarding the use of the Final Rule for the clinical trial was fueled by critics who argued that because trauma centers have the ability to provide allogenic blood, the experimental group should have received the available allogenic blood as standard of care and should not have been limited to 6u of PolyHeme first during the first 12 hours after injury. However, this criticism is based on the *assumption* that allogenic blood transfusions are an acceptable standard of care, and that PolyHeme's clinical indication was targeted only for use when red blood cells are not available. National media coverage failed to understand the purpose of the 12 hour time period which was necessary to simulate a delay in access to allogenic blood, a problematic scenario in military and rural environments, but not common to urban level one trauma centers. In this study the median time to arrival at the treatment facility for both cohorts was 26 min [23], reflecting robust trauma care systems and resource abundant environments. Had the treatment phase been limited only to prehospital care and all patients were to immediately receive allogenic blood upon arrival at a treatment facility within 26 minutes, the data would have been confounded by the ability of a resource rich environment to provide sufficient blood to all patients in a timely fashion. Additionally, the secondary endpoints of evaluating PolyHeme's potential to reduce allogenic blood transfusions and the incidence of MOF overall would not have been feasible had all patient's been eligible to received allogenic blood as early as 26 minutes after injury. Unfortunately, national coverage regarding the use of the waiver of consent

clouded the scientific rationale of the study design. The study protocol based on the 12 hour treatment phase was ultimately deemed a successful and ethically sound design to evaluate PolyHeme's ability to serve as an acceptable Hb carrying substitute when allogenic blood is not readily available.

Lastly, the failure to meet the prespecified statistical marker for the noninferiority analysis of 30 Day mortality raises questions regarding the appropriate endpoint of novel trauma interventions intended for prehospital or even early in hospital use. Field resuscitation interventions should ideally prolong survival so that patients will reach the definitive medical treatment facility, but these interventions may have limited effect on long term survival when not viewed in the context of the appropriate patient population. The severity of trauma injuries can be broken down into 3 categories: survivable injuries regardless of treatment, nonsurvivable injuries regardless of treatment, and potentially survivable injuries in which the use of timely, critical interventions will determine the survival outcome [17]. Evaluating novel interventions in the first two categories of patients will not provide the appropriate cohort to conclude efficacy. In the phase III clinical trial the overall mortality rate was only 13% (86/714), and half of the control group did not require any blood components in the first 12 hours [23], reflecting a population in which PolyHeme would not be indicated for. Thus, the entire study cohort included in the Per Treatment analysis may not have been an accurate reflection of the target population that could benefit from the early use of PolyHeme. A post-hoc analysis performed after the clinical trial showed that PolyHeme subjects had significant prolonged survival during the first 8 hours postinjury (Fig. 30.2) [17]. Perhaps a more targeted primary endpoint such as time to survival may have been a better marker of efficacy for the PolyHeme product's intentions; however, the ideal end point for specific trauma interventions continues to remains a controversial topic with the FDA and trauma researchers today [27].

While no Hb blood substitutes have yet been FDA approved for treatment of trauma patients in the US the future remains hopeful. There is currently a Department of Defense supported trial underway in South Africa evaluating the use of Hemopure®, a bovine Hb solution manufactured by Hemoglobin Oxygen Therapeutics LLC (Souderton, PA). Hemopure® is already approved in South Africa for treating anemia in adult surgical patients, however this clinical trial will enroll on 1400 trauma patients who would be eligible to receive Hemopure® in the prehospital setting [28]. Perhaps this clinical trial can avoid the pitfalls that plagued the PolyHeme USA prehospital trial and perhaps Hb blood substitutes will once again find themselves on US ambulances.

Summary

PolyHeme's brief 25 year development period can serve as lessons learned for future novel trauma interventions on the road to FDA approval. While the clinical trials supported the safe, effective use of PolyHeme to delay the need for and decrease the total number of allogenic blood transfusions needed in trauma patients, the product was ultimately unsuccessful in achieving FDA clearance after failing to meet a prespecified 30 day mortality rate. There still remains a significant need for not only oxygen carrying red blood cell alternatives

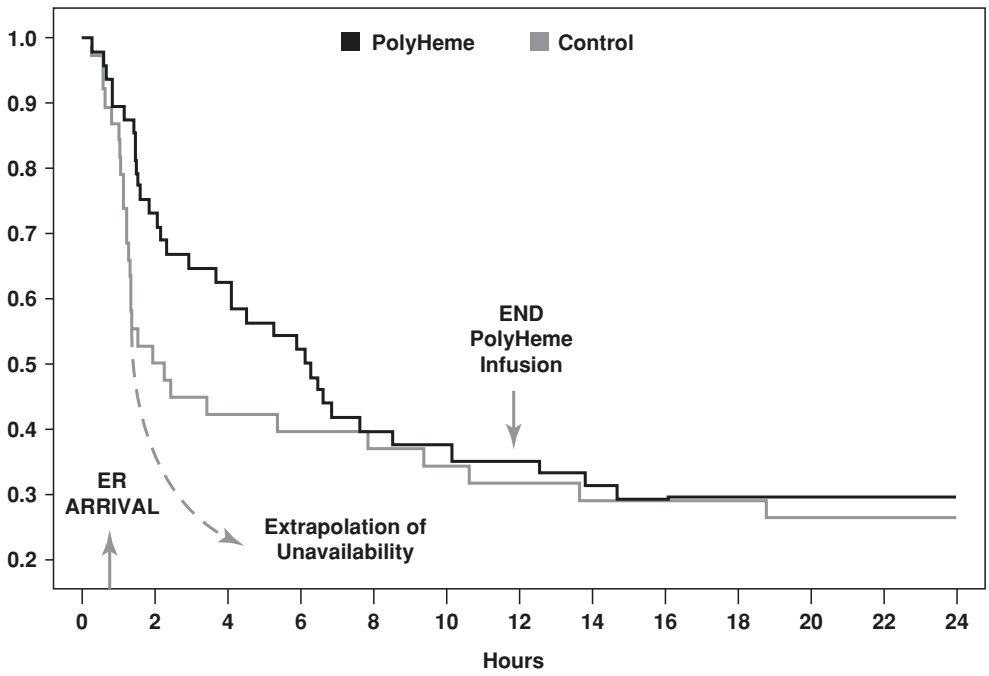


Fig. 30.2 Kaplan Meyer Curve showing time to death in non-surviving patients who received Polyheme or control. (From Bernard et al [17]; with permission)

products, but any interventions or products that can prolong prehospital survival in severely injured trauma patients who require definitive, but potentially delayed medical care. Yet these scenarios, such as military casualties, mass casualties, or environmental disaster settings are not easily incorporated into the clinical trial processes and trauma researchers must continue to develop novel clinical trial protocols similar to the PolyHeme trials that allow the successful extrapolation of results to these specific populations.

Key Points

- PolyHeme was the first Hb product intended for use in trauma patients when RBC would be unavailable
- 500 mL of PolyHeme is comparable to 1 unit of pRBC, providing 10 g/dL of Hb
- In the Phase II Clinical trial to historical controls subjects who received PolyHeme in lieu of pRBCs and had RBC [Hb] <1 g/dL had a survival rate of 75%
- When compared to pRBCs, PolyHeme results in less cytotoxicity and proinflammatory side effects, potentially reducing the risk of MOF in trauma patients
- The Phase III Clinical trial testing PolyHeme in the prehospital setting ultimately failed to meet the prespecified noninferiority goal of no more than 7% higher mortality than the control group, resulting in rejection of its FDA approval

References

1. Chen JY, Scerbo M, Kramer G. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. Clinics (Sao Paulo). 2009;64:803–13.
2. Sehgal LR, Gould SA, Rosen AL, Sehgal HL, Moss GS. Polymerized pyridoxylated hemoglobin: a red cell substitute with normal oxygen capacity. Surgery. 1984;95:433–8.
3. Gould SA, Sehgal LR, Rosen AL, Sehgal HL, Moss GS. The development of polymerized pyridoxylated hemoglobin solution as a red cell substitute. Ann Emerg Med. 1986;15:1416–9.
4. Gould SA, Sehgal LR, Sehgal HL, Moss GS. The development of hemoglobin solutions as red cell substitutes: hemoglobin solutions. Transfus Sci. 1995;16:5–17.
5. Gould SA, Moss GS. Clinical development of human polymerized hemoglobin as a blood substitute. World J Surg. 1996;20:1200–7.
6. Moss GS, Gould SA, Sehgal LR, Sehgal HL, Rosen AL. Hemoglobin solution--from tetramer to polymer. Surgery. 1984;95:249–55.
7. Savitsky JP, Doczi J, Black J, Arnold JD. A clinical safety trial of stroma-free hemoglobin. Clin Pharmacol Ther. 1978;23:73–80.
8. Moss GS, Gould SA, Sehgal LR, Sehgal HL, Rosen AL. Hemoglobin solution--from tetramer to polymer. Prog Clin Biol Res. 1984;165:191–210.
9. Sehgal LR, Rosen AL, Gould SA, Sehgal HL, Moss GS. Preparation and in vitro characteristics of polymerized pyridoxylated hemoglobin. Transfusion. 1983;23:158–62.
10. Gould SA, Sehgal LR, Rosen AL, Sehgal HL, Moss GS. The efficacy of polymerized pyridoxylated hemoglobin solution as an O2 carrier. Ann Surg. 1990;211:394–8.
11. Gould SA, Rosen AL, Sehgal LR, Sehgal HL, Moss GS. Polymerized pyridoxylated hemoglobin: efficacy as an O2 carrier. J Trauma. 1986;26:903–8.
12. Gould SASL, Sehgal HL, Toyoke E, Moss GS. Clinical experience with human polymerized hemoglobin. Transfusion. 1993;33
13. Apte SS. Blood substitutes--the polyheme trials. Mcgill J Med. 2008;11:59–65.
14. Gould SA, Moore EE, Moore FA, Haenel JB, Burch JM, Sehgal H, Sehgal L, DeWoskin R, Moss GS. Clinical utility of human polymerized hemoglobin as a blood substitute after acute trauma and urgent surgery. J Trauma. 1997;43:325–31. discussion 31-2.
15. Gould SA, Moore EE, Hoyt DB, Burch JM, Haenel JB, Garcia J, DeWoskin R, Moss GS. The first randomized trial of human polymerized hemoglobin as a blood substitute in acute trauma and emergent surgery. J Am Coll Surg. 1998;187:113–20. discussion 20-2.
16. Gould SA, Moore EE, Hoyt DB, Ness PM, Norris EJ, Carson JL, Hides GA, Freeman IH, DeWoskin R, Moss GS. The life-sustaining capacity of human polymerized hemoglobin when red cells might be unavailable. J Am Coll Surg. 2002;195:445–52. discussion 52-5.
17. Bernard AC, Moore EE, Moore FA, Hides GA, Guthrie BJ, Omert LA, Gould SA, Rodman GH Jr, PolyHeme SG. Postinjury resuscitation with human polymerized hemoglobin prolongs early survival: a post hoc analysis. J Trauma. 2011;70:S34–7.
18. Moore EE, Moore FA, Harken AH, Johnson JL, Ciesla D, Banerjee A. The two-event construct of postinjury multiple organ failure. Shock. 2005;24(Suppl 1):71–4.
19. Moore FA, Moore EE, Sauaia A. Blood transfusion. An independent risk factor for postinjury multiple organ failure. Arch Surg. 1997;132:620–4. discussion 4-5.
20. Moore EE. Blood substitutes: the future is now. J Am Coll Surg. 2003;196:1–17.
21. Johnson JL, Moore EE, Offner PJ, Partrick DA, Tamura DY, Zallen G, Silliman CC. Resuscitation with a blood substitute abrogates pathologic postinjury neutrophil cytotoxic function. J Trauma. 2001;50:449–55. discussion 56.
22. Johnson JL, Moore EE, Gonzalez RJ, Fedel N, Partrick DA, Silliman CC. Alteration of the postinjury hyperinflammatory response by means of resuscitation with a red cell substitute. J Trauma. 2003;54:133–9. discussion 9-40.
23. Moore EE, Moore FA, Fabian TC, Bernard AC, Fulda GJ, Hoyt DB, Duane TM, Weireter LJ Jr, Gomez GA, Cipolle MD, et al. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. J Am Coll Surg. 2009;208:1–13.
24. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA. 2008;299:2304–12.
25. Moore EE, Johnson JL, Moore FA, Moore HB. The USA Multicenter Prehosptial hemoglobin-based oxygen carrier resuscitation trial: scientific rationale, study design, and results. Crit Care Clin. 2009;25:325–56, Table of Contents.
26. Dougherty AH. In defense of the PolyHeme trial. Am J Bioeth. 2006;6:W35–7.
27. Holcomb JB, Moore EE, Sperry JL, Jansen JO, Schreiber MA, Del Junco DJ, Spinella PC, Sauaia A, Brohi K, Bulger EM, et al. Evidence-based and clinically relevant outcomes for hemorrhage control trauma trials. Ann Surg. 2021;273:395–401.
28. HBOC-201® To Be Evaluated In Groundbreaking Clinical Study Of Trauma Patients. PRNewswire. (Accessed 29 March, 2021. https://www.hbo2therapeutics.com/post/HBOC-201-to-be-evaluated-in-groundbreaking-clinical-study-of-trauma-patients-in-the-prehospital-set).

31

Clinical Evaluation of MP4CO: A Phase 1b Escalating-Dose, Safety and Tolerability Study in Stable Adult Patients with Sickle Cell Disease

Peter E. Keipert
and For the MP4CO-SCD-105 Study Investigators

Introduction

The hallmark of Sickle Cell disease (SCD) remains painful vaso-occlusive crises (VOC), yet to date no agent has been approved to treat these acute ischemic events [1]. MP4CO, developed by Sangart Inc. (San Diego, CA), is a hemoglobin-based carbon monoxide (CO) delivery agent and oxygen therapeutic that has shown potential in non-clinical studies to prevent and reverse red blood cell (RBC) sickling. MP4CO has exhibited anti-adhesive, anti-inflammatory, anti-oxidant, and anti-apoptotic properties at circulating CO-hemoglobin levels <10 % [2]. These protective effects are expected to limit progression of vascular occlusion and mitigate the consequences of ischemic tissue damage and inflammation. The beneficial effects of MP4CO have been ascribed to five mechanisms: (1) down-regulation of ICAM-1, VCAM-1 and NF-kB, (2) upregulation of Heme-Oxygenase-1 and Nrf2 leading to increases in anti-inflammatory mediators, biliverdin and CO, (3) delivery of oxygen to ischemic tissues, (4) intravascular volume expansion and improved perfusion of ischemic tissue, and (5) potential to prevent and reverse polymerization and sickling of RBCs [3, 4].

The investigational product, MP4CO, is pegylated human hemoglobin saturated with CO, and formulated at 4.3 g/dL in physiological acetate electrolyte solution. MP4CO is hyperoncotic (colloid osmotic pressure [COP] ~70 mmHg) with a high affinity for oxygen (P50 ~5 mmHg), resulting in the release of oxygen at low pO_2 to target delivery of oxygen in capillaries and ischemic tissues [5]. The aim of this Phase 1b first-in-man study was to evaluate safety of escalating-doses of MP4CO in adult patients with Sickle Cell disease not experiencing a painful VOC at the time of treatment [6].

Methods

We conducted a double blind, comparator controlled, dose-escalation, multi-center Phase 1b study at five sites in four countries [7]. Adult Sickle Cell patients with HbSS or S/β^0 Thal genotype, at least 18 years of age, who were clinically stable (not experiencing a VOC at the time of testing) and met all eligibility criteria were randomized to receive either MP4CO or normal saline (NS) in a sequential series of six escalating dose cohorts (A–F). In each cohort, three patients received MP4CO (Treatment) and one patient (Control) received normal saline (NS) to maximize the number of treated patients receiving investigational product. Single IV dose cohorts A–D received: (A) 15 or (B) 43 mg/kg (0.35 or 1.0 mL/kg) at 25 mL/min, (C) 86 or (D) 172 mg/kg (2.0 or 4.0 mL/kg) infused over 2 h. Cohorts E and F received fractionated doses totaling 172 or 344 mg/kg (4.0 or 8.0 mL/kg), administered as two IV infusions of 2.0 or 4.0 mL/kg given 24 h apart.

Safety assessments occurred at 24 (±4 h), 48 (±4 h), and 72 h (±1 day) after dosing, followed by visits at Day 7 (±1 day) and a safety follow-up at Day 28 (+5 days). Serious adverse events (SAEs) were monitored until Day 28. Safety evaluations included physical examinations, vital signs, pulse oximetry, Holter ECGs, transesophageal echocardiography (TEE) to measure tricuspid regurgitation jet velocity (TRJV) for estimating pulmonary artery systolic pressures, venous blood co-oximetry, free plasma hemoglobin, pain levels by Visual Analogue Scale (VAS), lab assessments (blood chemistry and hematology), and adverse events (AEs). Laboratory, hematology and chemistry parameters were summarized using descriptive statistics at baseline (BL) and at each post-BL time point. Safety data from base-

MP4CO-SCD-105 Study Investigators: Jo Howard, MB, BChir, Guy's and St. Thomas' Hospital NHS Foundation Trust, London, UK; Swee Lay Thein, MBBS, DSc, King's College Hospital NHS Foundation Trust, London, UK; Frédéric Galactéros, MD, PhD, Hôpital Henri-Mondor, Créteil, France; Adlette Inati, MD, Lebanese American University Hospital, Beirut, Lebanon; and Marvin Reid, MD, PhD, Sickle Cell Unit, University of the West Indies at Mona, Kingston, Jamaica

P. E. Keipert (✉) · For the MP4CO-SCD-105 Study Investigators
KEIPERT Corp. Life Sciences Consulting, San Diego, CA, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_31

line through Day 7 for all four patients enrolled within each cohort were reviewed by an unblinded, independent, medical monitor before proceeding to the next higher dose level.

Results

In total, 24 patients (of 25 randomized) were dosed, out of 35 screened. Patients were 54 % male, with an average age of 30 years (range: 18–51) and an average weight of 65 kg (range: 53–79). There were no clinically notable changes from BL, and no between-group differences observed in the clinical chemistry parameters evaluated, which included the following: albumin, liver enzymes (alanine amino-transferase [ALT], aspartate aminotransferase [AST], and gamma-glutamyl transferase [GGT]), pancreatic enzymes (amylase, lipase), electrolytes, glucose, serum bilirubin, creatinine, urea levels, and cardiac Troponin-I.

Overall, 16/24 patients (66.7 %) reported mild to moderate AEs; with 13/18 (72 %) in the MP4CO group vs. 3/6 (50 %) in NS Controls. The most frequent AE that appeared to be treatment-emergent was headache (see Table 31.1 for full listing of most common AEs reported in >2 patients). No SAEs were experienced, and no deaths occurred.

Vital signs, ECG readings, standard laboratory values and pulmonary pressures remained within normal limits. There was no evidence of hypertension due to vasoconstriction, based on systolic and diastolic blood pressure data (see Fig. 31.1).

A dose-related increase in venous CO-Hb levels was seen in the higher dose MP4CO cohorts (D–F), where CO-Hb increased by 1–2 % (absolute) after each dose and then normalized to pre-dosing levels by 8 h. There were no notable changes observed in methemoglobin levels. Low levels of free plasma hemoglobin were seen at baseline in all MP4CO groups and in NS controls, ranging from 0.08 to 0.13 g/dL. In MP4CO patients in cohorts C and D, plasma hemoglobin doubled at Hour 2, to mean levels of 0.20–0.35 g/dL. Peak plasma hemoglobin at 2 h after Dose 2 in MP4CO patients in cohort F reached a mean level of 0.41 g/dL.

Neither the MP4CO nor the NS group showed evidence of increased hemolysis, based on evaluating markers of hemolysis (i.e., total hemoglobin, reticulocyte count, lactate dehydrogenase [LDH] and total bilirubin). There were no notable between-group differences in mean values or ranges for hematological parameters, including WBCs and differentials (neutrophils, monocytes, lymphocytes, basophils and eosinophils), platelet counts, RBCs, hematocrit, and RBC morphology (MCH, MCHC, MCV).

There were no new treatment-emergent ECG abnormalities observed that were not already present at Baseline prior to dosing. There was no evidence of adverse cardiac effects or arrhythmias, and no exacerbation of any pre-existing abnormalities. TRJV results, assessed by noninvasive TTE, was similar in MP4CO and NS groups (see Fig. 31.2), suggesting the absence of any clinically significant increase in pulmonary artery systolic pressure after administration of MP4CO or NS.

There was no symptomatic or clinical evidence of renal dysfunction in either group based on serum creatinine and urine albumin levels. There were no notable dose effects seen, or any obvious between-group differences in urine albumin, urine β2-Microglobulin (β2M) and *N*-acetyl-β-$_D$-glucosaminidase (NAG) levels during the study (i.e., compared to mean values for all six Control patients combined). Two MP4CO-treated patients exhibited elevated levels of urinary β2M and NAG at Hour 72 that normalized at follow-up visits. Both patients also had intercurrent illnesses; one (cohort E) had an active mild VOC) during the study, and one (cohort F) was found to have influenza with pyrexia, rhinorrhea and cough. Both 72-h urine samples were analyzed further for Kidney Injury Molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) levels, which were found to be normal. This suggests that the elevations of β2M and NAG were likely due to the inflammatory processes (corroborated by elevated C-reactive protein [CRP] levels) and not specifically due to renal tubular injury.

The majority of the VAS measurements collected at screening, BL, post-dosing and daily thereafter were zero (no pain), or only negligibly elevated. Four patients had transiently elevated VAS pain scores. One NS control patient reported a VAS score of 37 mm (on a scale from 0 to 100

Table 31.1 Most common adverse events (reported in >2 patients)

Adverse event: preferred term	MP4CO (n = 18) T[a]	n	NS (n = 6) T[a]	n	Total (N = 24) T[a]	N
Headache	17	9 (50 %)	1	1 (17 %)	18	10 (42 %)
Fatigue	10	7 (39 %)	2	1 (17 %)	12	8 (33 %)
Rash (Holter application sites)	5	5 (28 %)	0	0	5	5 (21 %)
Back pain	10	3 (17 %)	0	0	10	3 (13 %)
Dizziness	3	3 (17 %)	0	0	3	3 (13 %)
Nausea	3	3 (17 %)	1	1 (17 %)	4	4 (17 %)
Musculoskeletal chest pain	3	3 (17 %)	1	1 (17 %)	4	4 (17 %)
Pain in extremities	2	2 (11 %)	3	2 (33 %)	5	4 (17 %)
Arthralgia	4	2 (11 %)	1	1 (17 %)	5	3 (13 %)
Cough	2	2 (11 %)	1	1 (17 %)	3	3 (13 %)
Infections and Infestations	2	2 (11 %)	1	1 (17 %)	3	3 (13 %)

[a]T = Total # of reported cases for each AE. n = # of patients with at least one report of an AE. Percentages are based on number of patients in Safety Population/treatment group and in total N

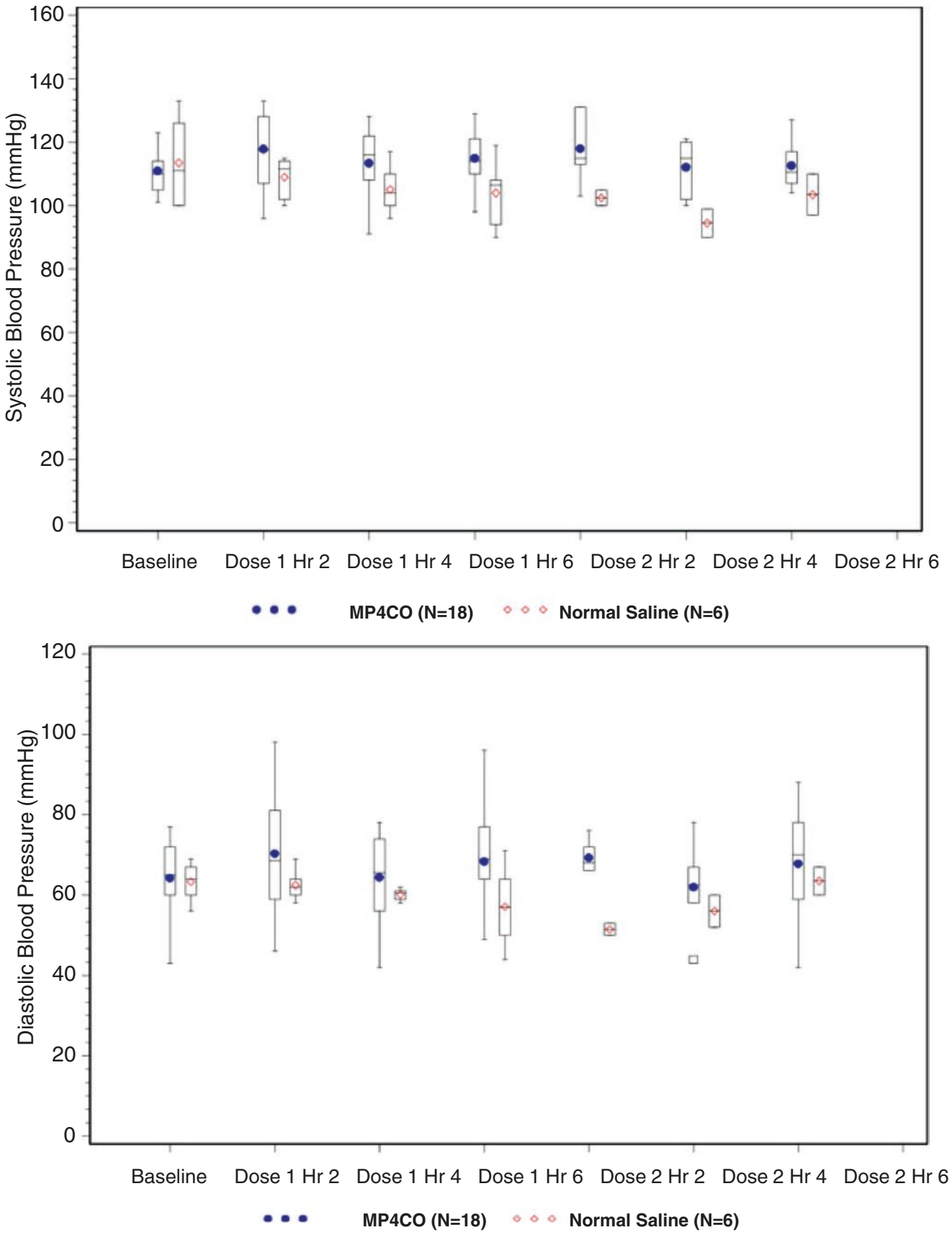


Fig. 31.1 Systolic and Diastolic blood pressure results (Box-and-Whisker plot). **Note**: Single and fractionated doses are combined

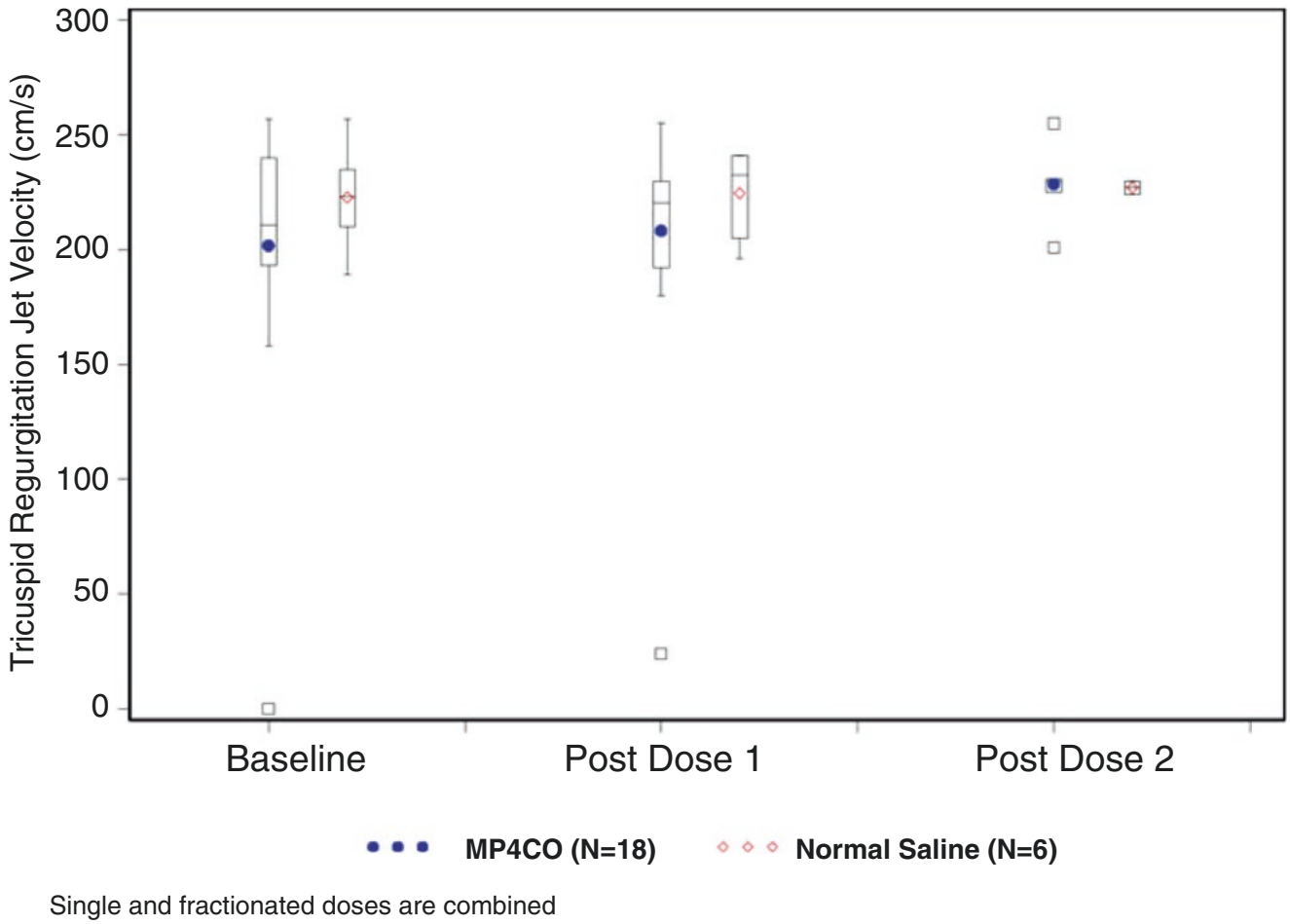


Fig. 31.2 Tricuspid regurgitation jet velocity (TRJV) results (Box-and-Whisker plot). **Note**: Single and fractionated doses are combined

mm) at 24 h after dosing. Similarly, one MP4CO-treated patient in cohorts D, E and F had VAS scores that briefly peaked to 30, 20 and 28 mm, respectively, during the study.

Discussion and Conclusions

The treatment of acute tissue ischemia associated with VOCs in patients with Sickle Cell disease represents an indication that may benefit from the unique attributes of MP4CO, which include: (1) rapid release of a small therapeutic dose of CO, (2) increased colloidal osmotic pressure to enhance capillary blood flow and tissue perfusion, (3) targeted oxygen delivery in the microcirculation and to ischemic tissues after release of the CO, (4) pegylation to prolong circulating half-life, and (5) lower concentration of hemoglobin to improve safety profile [2].

This study demonstrated minimal evidence of adverse effects of MP4CO on cardiac, pulmonary, hepatic, pancreatic and renal biomarkers, and no clinical evidence of any organ dysfunction or injury. Most AEs reported were consistent with events that are typically seen in patients with Sickle Cell disease. Unlike earlier generation hemoglobin-based oxygen carriers (HBOCs), neither vasoconstriction nor pulmonary hypertension was observed in this study. Taken together with data from non-clinical mechanism-of-action studies, these encouraging safety results suggest that MP4CO was well tolerated, and support further testing of MP4CO in a larger Phase 2 study for acute therapeutic intervention to treat painful VOCs.

Acknowledgments PEK was employed by Sangart Inc. as VP, Clinical Development, during the conduct of the MP4CO-SCD-105 study.

References

1. Ballas SK, Lusardi M. Hospital readmission for adult acute sickle cell painful episodes: frequency, etiology, and prognostic significance. Am J Hematol. 2005;79(1):17–25.

2. Belcher JD, Young M, Chen C, et al. MP4CO, a pegylated hemoglobin saturated with carbon monoxide, is a modulator of HO-1, inflammation, and vaso-occlusion in transgenic sickle mice. Blood. 2013;122(15):2757–64.
3. Belcher JD, Mahaseth H, Welch TE, et al. Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. J Clin Invest. 2006;116:808–16.
4. Winslow RM, Lohman J, Malavalli A, et al. Comparison of PEG-modified albumin and hemoglobin in extreme hemodilution in the rat. J Appl Physiol. 2004;97:1527–34.
5. Vandegriff KD, Young MA, Lohman J. CO-MP4, a polyethylene glycol-conjugated haemoglobin derivative and carbon monoxide carrier that reduces myocardial infarct size in rats. Br J Pharmacol. 2008;154(8):1649–61.
6. Howard J, Thein SL, Keipert PE. Poster presentation of MP4CO-SCD-105 study results at the American Society of Hematology (ASH) meeting. LA: New Orleans; 2013.
7. Howard J, Thein SL, Galactéros F, et al. Safety and tolerability of MP4CO: a dose escalation study in stable patients with Sickle Cell disease. Blood. 2013;122(21):2205.

Oxygent™, a Perfluorochemical-Based Oxygen Therapeutic for Surgical Patients

32

Peter E. Keipert

Introduction

For many decades, scientists have actively pursued development of 'artificial blood' – seeking a product that is safe, universally compatible with all blood types, and readily available. Despite the safety of allogeneic (donor) blood having improved significantly in recent years, the public's perception of the dangers still associated with allogeneic blood remains high, fueled primarily by documented cases of viral disease transmission, and fatal hemolytic transfusion reactions due to clerical errors. Furthermore, new viruses and prions continue to be discovered and publicized in the media, and any of these could end up in the blood supply, where they might become a new transfusion risk in the future.

Oxygen therapeutics (typically referred to as 'blood substitutes') are anticipated to play an important role in easing the increasingly frequent shortages of donor blood [40]. These agents would also avoid several transfusion-related safety issues, and could thereby profoundly change the practice of transfusion medicine in the future. Significant clinical development challenges remain, however, since regulatory approval of these products for a transfusion avoidance indication may require that they be proven to be as safe as allogeneic blood.

Emulsion Characteristics

For over 15 years, Alliance Pharmaceutical Corp. (San Diego, CA) was developing Oxygent™, a concentrated and stabilized second-generation perfluorochemical (PFC) emulsion. The optimized formulation contained 60 g PFC/dL (~31 per cent v/v) comprised of two active pharmaceutical ingredients; perflubron (perfluorooctyl bromide, $C_8F_{17}Br$) as the principal PFC and a small quantity of perflubrodec (perfluorodecyl bromide; $C_{10}F_{21}Br$) to stabilize particle size growth during storage [39]. To make Oxygent, the PFCs undergo emulsification using pharmaceutical-grade egg-yolk phospholipid (the same surfactant typically used to emulsify other commercial lipid-based emulsion products – such as Intralipid™) in a buffered electrolyte solution. Both PFCs used in Oxygent have been produced in large-scale (metric ton) quantities and are available at pharmaceutical-grade purity (> 99.99 per cent) from commercial suppliers. The oxygen solubility for the neat PFC used in Oxygent is ~53 mL/dL per atmosphere, resulting in a net oxygen transport carrying capacity of ~16 mL/dL per atmosphere for the 60 per cent w/v Oxygent emulsion formulation.

The manufacturing procedures and formulation for Oxygent developed and patented by Alliance comprise a simple, efficient, high-yield process that can produce emulsion particles with an average median diameter of 0.16–0.18 μm [5], approximately one-fortieth the size of a red blood cell. Oxygent was formulated in phosphate-buffered saline with an osmolality of 300–310 mOsm/kg, buffered to neutral pH (7.0–7.2), and had a viscosity of ~4 cPs (25 °C, shear of 1/s). Oxygent was terminally heat-sterilized (temperature > 120 °C) and was formulated as a ready-to-inject emulsion in 110-mL single-use glass vials sealed with a spikable rubber stopper and aluminum over seal. Oxygent had a 24-month shelf life when stored at normal refrigeration (2–8 °C), but the emulsion was stable enough to tolerate exposure at room temperature (25–30 °C) for up to several weeks.

Non-Clinical Safety

The safety of Oxygent was evaluated in over 250 preclinical studies in multiple animal species. Extensive toxicology studies demonstrated that Oxygent was well tolerated with no serious adverse effects at clinically relevant doses (approximately 1.0–6.0 mL/kg). These results were confirmed in several special safety-related toxicology studies performed to assess many different biological parameters,

P. E. Keipert (✉)
KEIPERT Corp. Life Sciences Consulting,
San Diego, CA, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_32

including systemic hemodynamics, blood components with emphasis on hemostasis and platelet function, immune function and host resistance, and pulmonary function. In addition, a series of ancillary pharmacology studies demonstrated no significant changes in behavioral or physiological evaluations, locomotor activity, gastrointestinal motility, cardiovascular or respiratory evaluations, neuromuscular function, or interference with the efficacy of various common anesthetic and analgesic drugs.

The most common biological effects observed following infusion of PFC emulsions in preclinical studies include (1) a short-lived febrile response starting several hours after dosing, and (2) a transient drop in platelet counts at 2–3 days post-dosing, but with no adverse effect on hemostasis (i.e., platelet function and bleeding time remain normal). The mechanisms for both of these effects have been elucidated, and are related to the normal clearance of the emulsion particles from the circulation by phagocytic cells (liver Kupffer cells and splenic macrophages) of the RES and to the physical properties of the emulsion, particularly particle size and the choice of surfactant [10]. The clearance of Oxygent particles from the circulation is dose-dependent, and yields a blood half-life of approximately 12 hours, with most of the drug being cleared from the blood within 48 hours [28]. Once cleared from the blood by macrophages, the surfactant is degraded, leaving behind the pure PFC. Since PFC molecules are completely inert they are not broken down in the body, reducing any potential for toxicity from metabolic degradation products. Instead, intact PFC molecules simply diffuse slowly from tissues back into blood, where they are transported, dissolved in blood lipids, and then eliminated from the body over time via expired air [40].

The rate of PFC elimination from tissues depends primarily on vapor pressure and lipid solubility of the PFC [53], and the time required to clear PFC from the tissues is essentially dose-dependent. Second-generation PFC emulsions based on perflubron used PFCs that are more lipid-soluble than the original PFCs (e.g. perfluorodecalin) used in earlier first-generation dilute PFC emulsions such as Fluosol® and Perftoran®. Both the incidence and the magnitude of biological effects stemming from the activity of macrophages and phagocytic RES cells are related to the emulsion formulation, especially with respect to the emulsion particle size – i.e., smaller-diameter (0.1–0.2 μm) particles tend to be less detectable by the RES and thereby attenuate the biological effects [25]. Complement activation was occasionally observed after administration of Fluosol due to its synthetic Pluronic® surfactant [20], but this was *not* observed with second-generation PFC emulsions like Oxygent that use egg-yolk lecithin as the only surfactant. Following administration of clinically relevant doses of Oxygent, there were no adverse hemodynamic effects (i.e., no vasoactivity) and no decreases in cardiac output, thereby allowing the PFC-dissolved oxygen to be effectively delivered to the tissues [21]. In contrast to blood or blood-derived products, there is also no risk of person-to-person disease transmission when using Oxygent, since it is free of any blood-derived components.

Clinical Safety

In total, almost 1,500 subjects were enrolled in 20 clinical studies with Oxygent, including Phase I healthy volunteer studies, and Phase II and III studies in surgical patients. Of these, more than 800 subjects received Oxygent. The safety profile of Oxygent was evaluated in several clinical studies involving healthy volunteers (four studies), cancer patients (six studies), general surgery patients (six studies), and cardiac surgery patients (four studies). Clinical studies with Oxygent in surgical patients tested doses ranging from approximately 1.5 mL/kg (0.9 g PFC/kg body weight) up to 6.0 mL/kg (3.6 g PFC/kg, which represents about four 110-mL units of Oxygent for an average 70-kg individual).

The overall safety of Oxygent was investigated in detail in Phase I studies with healthy volunteers. As reported by Leese et al. [30], a transient increase in body temperature was observed starting several hours after dosing in ~15 per cent of the treated awake subjects, but resolved within 12–24 hours. A mild transient decrease in total platelet count (< 20 per cent drop from starting levels) was observed at 3 days post-dosing, which recovered to baseline levels by 7 days. Despite this delayed effect on platelet count, this study and the one reported by Noveck et al. [34] also demonstrated the absence of any direct effect of Oxygent on platelet function (assessed by *ex vivo* platelet aggregation in response to agonists like arachidonic acid, collagen and ADP), and no prolongation of measured bleeding times or adverse impact on coagulation parameters [30]. In addition, there was no evidence of complement activation or immunogenic reactions; no suppression of humoral or cell-mediated immune function; no abnormal changes in liver, pulmonary, or renal function; no clinically meaningful effects on blood chemistry; and no hemodynamic effects or signs of vasoconstriction – i.e., no blood pressure or heart rate changes [34].

Preclinical Efficacy

The method of oxygen transport by PFCs is different from hemoglobin. Under normal conditions, about 20–30 per cent of the oxygen bound to Hb inside red cells is unloaded systemically and consumed by tissues. In contrast, Oxygent transports oxygen dissolved in the PFC droplets in proportion to the partial pressure of oxygen in the blood ($P\ O_2$), which can be substantially increased as the fraction of

inspired oxygen (F_iO_2) that the patient is breathing is elevated.

Hence, extraction of dissolved oxygen from a PFC emulsion is linear, and can exceed 90 per cent assuming an arterial PO_2 level of 500 mmHg [23]. As the Oxygent emulsion perfuses the microcirculation, dissolved oxygen is released initially from the PFC and the plasma, leaving a greater reserve of oxygen bound to the Hb inside the red blood cells; this makes Oxygent an attractive drug for a variety of clinical applications in which tissues may be at risk of acute hypoxia due to ischemia or transient anemia. This intrinsic property enables Oxygent to enhance tissue oxygenation even when administered in relatively small doses. Numerous preclinical studies have demonstrated that: (1) Oxygent supports tissue oxygenation; (2) the oxygen delivered by Oxygent is available to support metabolic processes at the tissue level; and (3) this improved oxygenation status translates into improved organ function [11].

Potential Clinical Applications

Tissue Oxygenation and Hemodilution

In normal animals under hyperoxic conditions, dosing with Oxygent was associated with a significant improvement in oxygen delivery and oxygen consumption in a dog model of maximally working isolated skeletal muscle [18], and with an increase in plasma oxygen solubility and tissue $P\ O_2$ in both dog skeletal muscle [13] and cat retina [3] even when using low doses of Oxygent (e.g. 0.9 g PFC/kg). Oxygent was shown in various studies to deliver oxygen to support metabolic processes, which correlated with improved organ function in critical tissues, including brain and heart. In the brain of normal awake rabbits [50] and anesthetized cats [35], Oxygent dosing markedly enhanced cerebral cortical $P\ O_2$ above levels achieved with oxygen-breathing alone. In a model of partial brain stem ischemia in dogs using transient basilar artery occlusion following dosing with Oxygent, Guo et al. [14] demonstrated a significant improvement in cerebral metabolic status and recovery of brain function (assessed by auditory evoked potentials) in the Oxygent-treated group.

Several studies were performed using canine hemodilution models designed to mimic acute surgical anemia and blood loss [11]. These studies employed the use of a number of oxygen electrodes to monitor $P\ O_2$ levels in heart, brain, muscle, gut, and liver tissue to assess the impact of Oxygent-induced blood $P\ O_2$ increases on tissue oxygenation. Results clearly showed that improvements in tissue oxygenation with Oxygent treatment correlated with the observed increases in blood oxygenation [23].

Studies were conducted in splenectomized dogs that were hemodiluted to a Hb concentration of 7–8 g/dL, administered Oxygent (doses from 0.9–5.4 g PFC/kg), and then subjected to volume-compensated blood loss to mimic intra-operative bleeding with fluid replacement [1, 15]. In each of these studies, overall oxygenation status was significantly improved during blood loss in animals given Oxygent compared to controls, while breathing either room air or 100% oxygen. Throughout the bleeding phase, Oxygent maintained adequate systemic oxygenation at lower Hb concentration levels than was possible without Oxygent, and preserved local tissue oxygenation as assessed by PO_2 electrode measurements in the gut [26] and in skeletal muscle and liver [1]. In particular, there was a positive correlation between tissue $P\ O_2$ and mixed venous oxygen tension ($P\ v^-O_2$) in this study [27]. As reported by Habler et al. [16], even after considerable hemorrhage, one dose of Oxygent (1.8 g PFC/kg) was as effective as about 3.8 g/kg of hemoglobin given as autologous blood in preserving adequate tissue $P\ O_2$ and maintaining better myocardial function as evidenced by improved left ventricular contractility. In addition, these studies demonstrated no untoward hemo-dynamic effects associated with the administration of Oxygent. Collectively, these non-clinical efficacy studies in surgical hemodilution models provided compelling evidence that Oxygent can prevent tissue hypoxia, and can preserve myocardial and cerebral function in the presence of acute anemia or ischemia.

Cardiopulmonary Bypass

Studies were also performed using canine models of cardiopulmonary bypass (CPB) to assess systemic and myocardial oxygenation parameters. Oxygent treatment resulted in a significant increase in mixed venous blood $P\ O_2$ ($P\ v^-O_2$) levels (an indirect reflection of tissue oxygenation) versus controls, in the absence of any changes in total oxygen consumption or hemodynamics [7]. Treatment was also associated with improved myocardial recovery post-bypass, as well as increased delivery and extraction of dissolved oxygen. In a study by Holman et al. [19], in anemic (hemodiluted) dogs put on bypass, the additional dissolved oxygen provided by Oxygent appeared to be responsible for ameliorating post-CPB cardiac function after weaning from bypass and resulted in a lower mortality rate in the Oxygent-treated group.

During CPB, gaseous micro-emboli can be created from multiple sources (cannulation, venting of the heart, oxygenator) and may be partly responsible for the neurological and neuropsychological deficits often observed in patients after cardiac surgery [44]. Pretreatment with Oxygent has provided benefits in terms of survival and recovery from transient neurological deficits in animal models. This protective effect may be due to a combination of the PFC's ability to resorb gaseous air micro-emboli (since nitrogen, the main

constituent of air, is ~25 times more soluble in PFC than in water at 37 °C) and to deliver oxygen and improve perfusion of ischemic regions in the brain. A canine model of transient brainstem ischemia was able to demonstrate full functional recovery of auditory evoked potentials in Oxygent-treated dogs compared to saline controls [14]. These data collectively suggest that it may be possible in the future to use Oxygent to prevent tissue injury arising from gaseous emboli in the blood during cardiac surgery, and potentially to ameliorate cerebral injury from ischemic hypoxia during neurosurgery (e.g. aneurysm clipping).

Shock and Trauma

A number of preclinical studies demonstrated the potential for future applications of Oxygent in a variety of critical care settings. In a study of endotoxin-induced shock in a canine model, Cain et al. [4] showed that Oxygent treatment increased oxygen uptake in the gut and in muscle, and improved regional blood flow and cardiac output. Using a model of hypotensive resuscitation from severe uncontrolled hemorrhage in swine breathing 33% to 67% oxygen, Stern et al. [46] demonstrated enhanced oxygen delivery and improved survival with Oxygent. In another dog model of cardiac arrest, aortic arch perfusion with Oxygent was shown to result in improved coronary perfusion and faster return of spontaneous circulation [31].

Resuscitation of hemorrhagic shock in two rat models with Oxygent demonstrated an improvement in cerebral oxygenation [52] and better restoration of hepatic energy metabolism [37]. A study by Daugherty et al. [6] analyzed the efficacy of using Oxygent in a rodent model of traumatic brain injury (TBI) using lateral fluid percussion. Results demonstrated that Oxygent treatment significantly increased cerebral oxygenation after TBI and enhanced mitochondrial function at 4 hours after injury as compared with saline controls.

Decompression Sickness

Because of their high solubility for all gases, including nitrogen in air, PFCs have been proposed as a potential treatment for decompression sickness [45]. Using a swine model of severe decompression sickness (DCS) and post-dive treatment with Oxygent, Dromsky et al. [8] demonstrated that Oxygent-treated animals sustained significantly less DCS than the controls (53 per cent vs 93 per cent), and no animals in the Oxygent group sustained neurological DCS, which was present in 69 per cent of the swine in the other two groups. These preliminary findings suggest a potential future indication for using PFC emulsions to treat DCS when hyperbaric treatment is delayed or unavailable.

Organ Preservation

Oxygent has undergone preclinical evaluation for preserving tissues and prolonging the storage time of an organ (e.g. kidney) prior to transplantation. Studies by Brasile et al. [2] demonstrated the ability to preserve canine kidney autografts without the need for extreme hypothermia, by pulsatile perfusion using Oxygent-supplemented media at 32 °C. Studies using isolated Langendorff rabbit heart preparations by Symons et al. [47] demonstrated that Oxygent supplementation of the perfusion media resulted in better maintenance of oxygen delivery, increased tissue oxygenation and high-energy phosphates, and improved myocardial function following low flow ischemia.

Tumor Oxygenation

Another application that has been studied extensively in different preclinical animal models bearing various implanted tumors involves the use of PFC emulsions for augmenting PO_2 levels in hypoxic tumors to enhance the tumor's sensitivity to radiation and chemotherapy [41, 48, 49]. Many of these preclinical studies demonstrated the basic efficacy of this approach, and this eventually led to preliminary clinical studies with Fluosol in oncology patients [9]. To date, however, no company has chosen to pursue this indication as their first commercial application for a PFC-or Hb-based oxygen therapeutic, perhaps because of the challenging regulatory requirement to demonstrate improved mortality in order to gain approval for such an application in treating cancer patients.

Sickle Cell Disease

Finally, the vaso-occlusive crises that commonly occur in sickle-cell disease patients may present another future application where the immediate oxygenation benefit provided by Oxygent and other oxygen therapeutics might eventually prove beneficial [38]. For example, Kaul et al. [22] demonstrated the efficacy of using Oxygent treatment to reduce sickle cell RBC-induced vaso-occlusion in the *ex vivo* mesocecum vasculature of the rat.

Phase II Clinical Studies

General Surgery

Several Phase II studies using Oxygent in general surgery were completed and enrolled approximately 250 subjects (primarily orthopedic, urologic and gynecologic patients).

Oxygent was well tolerated in these studies. In the first Phase IIa pilot study, the use of Oxygent was shown to increase blood oxygenation parameters (including mixed venous blood $P\ O_2$) in the absence of any adverse hemodynamic changes [51]. In two large multicenter studies, Oxygent was dosed in conjunction with augmented-acute normovolemic hemodilution (A-ANH). This technique typically involved the collection of several (2–4) units of the patient's autologous blood just prior to surgery, administration of Oxygent to replace the oxygen-carrying capacity of the harvested blood and blood lost during surgery, and later reinfusion of the collected autologous units when the surgery was completed [24]. By performing A-ANH, the patient's blood is temporarily diluted so that fewer red blood cells are actually lost during surgical bleeding.

As reported by Spahn et al. [42], the European Phase II general surgery study in orthopedic surgery patients (n = 147) clearly demonstrated the drug activity of Oxygent in terms of rapidly enhancing the patient's systemic oxygenation status and being able to effectively reverse physiological transfusion triggers – i.e., protocol-defined physiological parameters indicating the patient's need for a blood transfusion. This study demonstrated a significant prolongation in the duration of trigger reversal in the Oxygent-treated patients, thereby effectively delaying the need for transfusion of allogeneic blood. Similar findings were found in a parallel US study performed in urologic and gynecologic surgery patients (n = 99), as reported in abstract form by Monk et al. [32, 33].

Cardiac Surgery

Three small Phase II studies in cardiac surgery with Oxygent enrolled ~80 patients undergoing coronary artery bypass grafting (CABG) procedures with CPB. In these studies a blood harvesting technique known as intraoperative autologous donation (IAD) was performed by the perfusionist just as bypass was initiated. By implementing aggressive pre-bypass harvesting of autologous blood in the Oxygent-treated patients, Hill et al. [17] were able to demonstrate that Oxygent used in conjunction with IAD had the ability to prevent physiological transfusion triggers during bypass. More importantly, about 83 per cent of patients receiving the higher dose (2.7 g PFC/kg) of Oxygent were able to avoid transfusion of allogeneic blood completely (versus less than 45 per cent of control patients). By being able to tolerate a greater degree of autologous blood harvesting in the high-dose Oxygent group (~1600 mL vs ~990 mL in controls), these patients exhibited a trend (not significant due to the small number of patients per group) for reduction in the allogeneic blood transfusion requirements from an average of 1.8 units/patient (range 0–7 units) in controls to only 0.4 units/patient (range 0–3 units).

Phase III Clinical Studies

General Surgery

A multicenter Phase III study in 492 patients undergoing orthopedic, urologic, abdominal, vascular and other major surgical procedures (often to treat malignant disease) was conducted at 34 medical centers in 8 European countries. As reported by Spahn et al. [43], treated patients receiving Oxygent in conjunction with the A-ANH procedure avoided the need for blood transfusion more frequently than controls, and also required fewer units of allogeneic blood. The primary endpoint (reduction in RBC units transfused at 24 hours) was achieved in the intent-to-treat population (all randomized patients): the Oxygent group received 26 per cent fewer allogeneic units, 1.5 vs 2.1 units in controls (median 0 vs 1 unit; $P = 0.013$). By hospital discharge, the Oxygent group had received ~15 per cent fewer allogeneic units, (mean 2.7 vs 3.2 units; median of 1 vs 2 units), but this difference was no longer significant ($P = 0.16$). However, in the protocol-defined target population, i.e., patients with an estimated blood loss (EBL) of at least 20 mL/kg (n = 330 or 67 per cent of randomized subjects), the Oxygent group required less RBC units (mean 2.0 versus 3.3 units; median 1 vs 3 units; $P < 0.001$) on postoperative day (POD) 1 (Table 32.1), and this difference remained significantly different from controls through day of discharge (DD) (mean 3.4 versus 4.9; median 2 vs 4 units; $P < 0.001$).

Regarding complete avoidance in the entire study population, ~53 per cent of patients in the Oxygent group avoided allogeneic blood transfusions compared to ~42 per cent of controls ($P < 0.05$) during the acute study period (24 hours). At later time points, more Oxygent-treated patients continued to avoid blood transfusions, but the difference versus controls was no longer significant. Again, however, in the protocol-defined target population (EBL of at least 20 mL/kg), a significantly ($P < 0.05$) greater (almost two-fold) percentage of patients avoided transfusion at all time points from postoperative day 1 (D1) through postoperative day 21 or hospital discharge (P21/DD), whichever occurred sooner (see Fig. 32.1).

For patients who had surgical blood losses ranging from ~10 mL/kg to >80 mL/kg (representing 86 per cent of all randomized subjects), the Oxygent benefit was highly statistically significant for both avoidance of blood ($P = 0.002$) and reduction in blood usage ($P < 0.001$), and this clinical benefit was maintained and remained statistically significant through 21 days or to the day of hospital discharge.

The safety profile in this study, evaluated by an independent Data Safety Monitoring Board (DSMB), was considered to be acceptable for further development of Oxygent, and the adverse events (AEs) observed were those expected following major elective surgery. The incidence of AEs was

Table 32.1 Number of units of blood transfused from postoperative day 1 through hospital discharge

Study day	Oxygent group		Controls		% Reduction		*P*-value
Target pts. (n = 330)	Mean[a] ± SD		Median	Mean[a] ± SD	Median	(Means)	
1	2.0 ± 4.0		1	3.3 ± 3.0	3	40.8	< 0.001
3	2.7 ± 3.3		2	4.1 ± 2.7	3	33.2	< 0.001
7	3.2 ± 3.0		2	4.6 ± 2.5	4	30.3	< 0.001
21 or DD[b]	3.4 ± 2.9		2	4.9 ± 2.4	4	30.3	< 0.001

[a]Mean adjusted for covariates (analysis of covariance) using a natural log transformation
[b]Whichever occurred sooner; DD is day of discharge

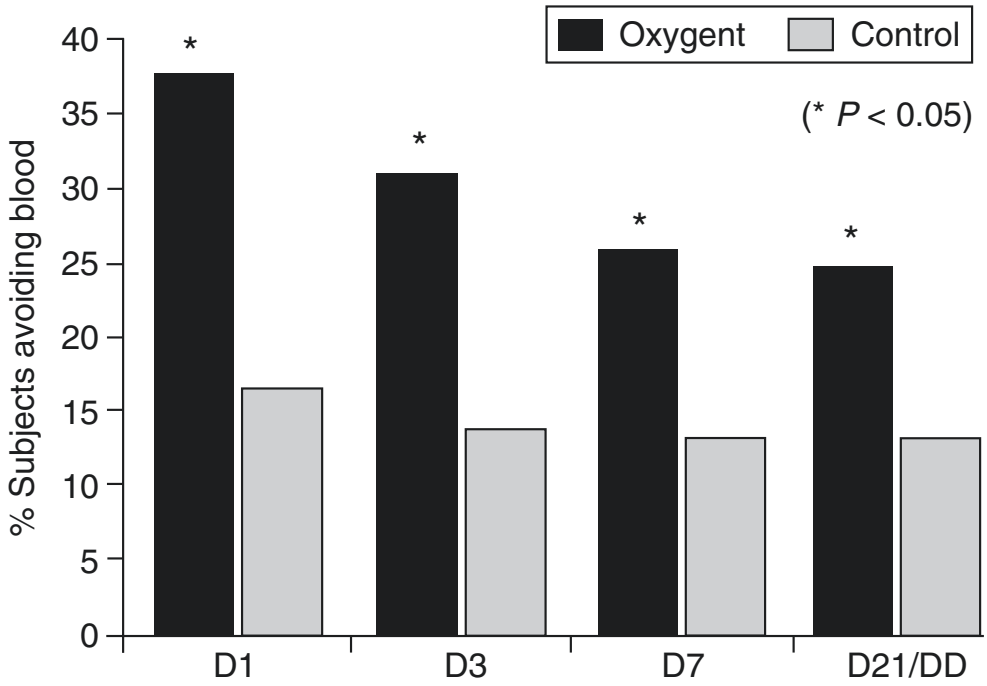


Fig. 32.1 Avoidance of allogeneic blood transfusion from postoperative day 1 (D1) through postoperative day 21 or hospital discharge (D21/DD), in the protocol-defined target population (patients with EBL of at least 20 ml/kg)

similar in the Oxygent group (86 per cent) compared to the control group (81 per cent). There was a higher overall incidence (~10 per cent differential) of serious adverse events (SAEs) in Oxygent-treated subjects (who had the A-ANH procedure) compared to controls (who did not). However, only the category 'Digestive system' was significantly different from control, mostly due to a higher reported occurrence of serious postoperative ileus (four instances in the Oxygent group versus none in controls). This reported 2 per cent incidence of ileus is rather low for large abdominopelvic operations, but surprisingly, the investigators did not report ileus in the controls – evidence that there was likely a reporting bias (i.e., in particular, underreporting of commonly encountered adverse events in the control group, since this was an unblinded study).

The DSMB monitoring this study did note group imbalances in certain adverse events, but concluded that there was no clinically consistent pattern or significance. They also concluded that since investigators were not blinded to treatment allocation, there was a possibility that this may have influenced reporting. The DSMB noted evidence that some investigators might not have adequately maintained normovolemia in the Oxygent-treated patients that were profoundly hemodiluted. Safety results from this study demonstrated that careful management of the patient's volume status and attention to optimal fluid balance is important to perform ANH safely. Overall mortality in this study was 3 per cent and the difference between groups (Oxygent 4 per cent vs controls 2 per cent) was not statistically significant. Tumor progression, sepsis and multiorgan failure, as well as typical surgical complications, were responsible for the deaths, and all were considered by the investigators to be due to underlying disease or conditions and were deemed to be unrelated to the study drug [43].

Cardiac Surgery

In parallel to the general surgery study described above, a Phase III cardiac surgery study was conducted in primarily US and Canadian centers to assess transfusion avoidance in CABG patients undergoing CPB. In January 2001, after

approximately 410 patients (of 600 intended) had been randomized, enrollment in this study was voluntarily suspended when a statistically significant imbalance developed in the incidence of stroke. During the initial analysis of the safety data from these patients, an imbalance in thoracic bleeding events requiring re-operation was also uncovered.

Adverse events were reported for all subjects enrolled in this study, as expected in a CABG patient population. Protocol-specified analyses showed no statistically significant differences in the overall incidence of AEs or SAEs between groups. The incidence of SAEs (34.4 per cent overall; Oxygent 38.5 per cent; control 30.3 per cent) and deaths (1.5 per cent overall; Oxygent 2.5 per cent; control 0.5 per cent) were generally within published ranges expected up to 3 months post-CABG surgery involving CPB, although the per cent mortality in the control group was exceptionally low. A cardiac surgery study published at that time by Klein et al. [29], involving a large cohort of patients undergoing CABG surgery with CPB, reported a mortality of 4.5 per cent, which is similar to the 3.8 per cent mortality published by Pan et al. [36] in a study of over 1,660 patients undergoing primary CABG surgery at the Texas Heart Institute between January 2000 and December 2001.

Although the overall intraoperative and postoperative safety findings in this study appeared to be acceptable, there was a significantly higher incidence of serious neurological events, including primarily strokes (2.4 per cent overall; Oxygent 5 per cent vs control 1 per cent). As it was for mortality, the incidence of neurological events in the controls was lower than expected for a CABG patient population. An imbalance in the incidence of serious postoperative thoracic chest tube bleeding requiring re-operation, a noted post-surgical complication after CABG surgery, also was observed (5.8 per cent overall; Oxygent 10 per cent vs control 1.5 per cent). Once again, the incidence of this SAE in the control group in this unblinded study was remarkably low compared to that reported in the literature. The overall incidence rates for the serious neurological and bleeding complications observed were within clinical expectations, and within ranges reported in the literature.

The etiology of the higher rates of these complications in subjects randomized to the Oxygent group compared to controls was the subject of extensive *post hoc* exploratory analyses and hypothesis generation. After detailed analyses of safety data from this study and all previous clinical studies, as well as additional laboratory evaluation of Oxygent in the presence of compounds that would come into contact with the emulsion in the bypass setting, no evidence was found to link Oxygent directly to the observed imbalances. Primary contributing factors for the adverse events appear to be the amount of autologous blood harvested and the way in which the rapid IAD procedure was performed in the Oxygent-treated group. In addition, there were imbalances in some of the risk factors for these events between the treatment and control groups, and a greater degree of dilutional coagulopathy and use of hetastarch in the Oxygent group. The result was that, in a subset of subjects at greater risk for complications, inadequate management of blood pressure during rapid blood harvesting potentially resulted in decreased perfusion to the brain. The conclusion that the procedure, rather than the product, was responsible for the adverse events was supported by the fact that there were no similar imbalances in the incidence of stroke in the three Phase II cardiac surgery studies (in which neurological and neuropsychiatric evaluations were performed in a blinded manner). No similar imbalance was seen in the Phase III general surgery studies, or in any previous clinical studies with Oxygent. All of these findings were summarized in a Clinical Information amendment and an Integrated Summary of Safety (ISS) and were subsequently provided to the FDA and to the European regulatory authorities (EMEA).

As reported by Frumento et al. [12], a small subset of patients in the Phase III cardiac surgery study were being monitored with gastric tonometry to assess the overall status of gut perfusion and oxygenation. As shown in Fig. 32.2a, a significant benefit was seen in the Oxygent-treated patients; the CO_2 gap (i.e., gastric CO_2 minus $PaCO_2$) was significantly lower ($P < 0.001$) and gastric pH was significantly higher ($P < 0.01$), suggesting improved perfusion of the gut. These benefits, both of which persisted throughout surgery, suggested better maintenance of gut microcirculatory oxygenation and translated into significantly ($P < 0.007$) faster return of normal bowel function in the postoperative period (Fig. 32.2b).

Future Clinical Development

Avoidance of allogeneic blood transfusion remains a highly desired clinical objective of both physicians and patients. Previous clinical studies in surgery have demonstrated that using Oxygent to augment the amount of autologous blood harvested could result in a significant decrease in allogeneic transfusion requirements, primarily in patients who lose at least 3 units of blood during surgery. However, careful analysis of safety data from these studies also indicated that ANH, and especially the rapid IAD just before cardiac bypass, must be performed very carefully, with close attention to optimal blood volume management to maintain euvolemia. Hence, based on the data from their previous Phase II and Phase III clinical trials, Alliance designed a new Phase III protocol without any autologous blood harvesting to further evaluate the efficacy and safety of Oxygent in a general surgery population. Oxygent would be administered when surgical bleeding had decreased the patient's hemoglobin to a level that results in a physiologic or Hb-based transfusion

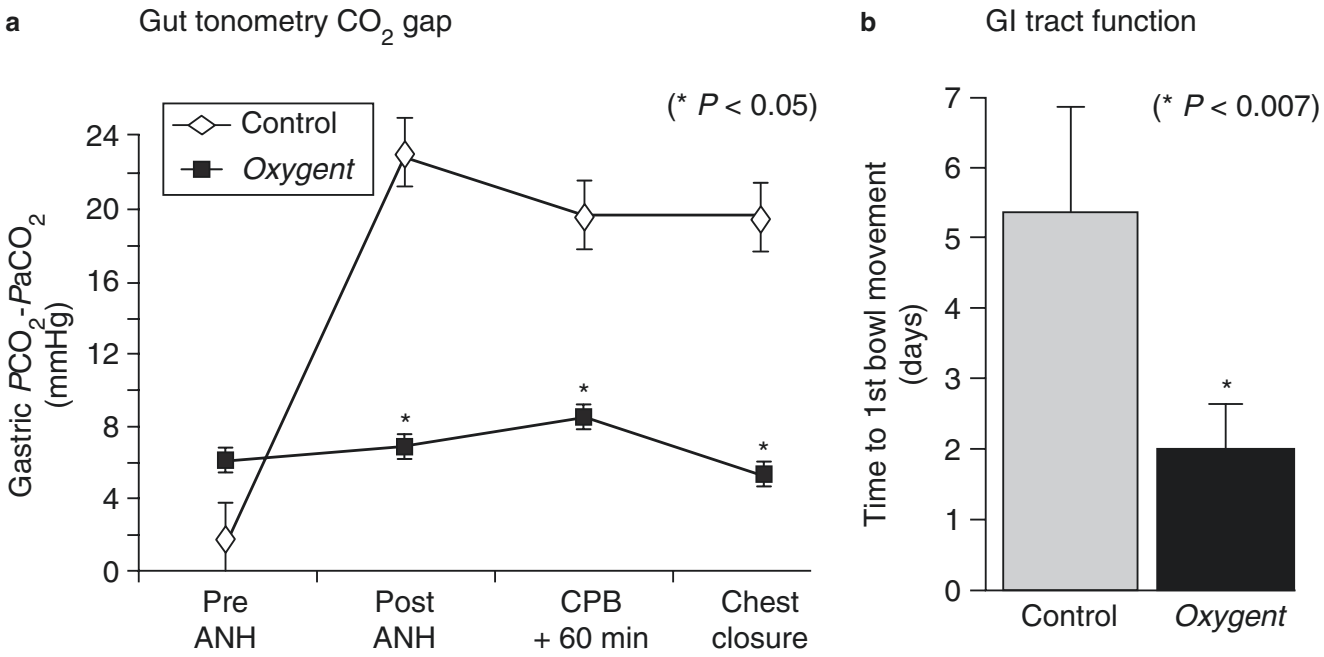


Fig. 32.2 Assessment of gut perfusion/oxygenation status and bowel function. (**a**) CO_2 gap (gastric CO_2 measured by tonometry minus $PaCO_2$). (**b**) Postoperative recovery of gut function determined by time to first bowel movement

trigger. Delaying Oxygent dosing until a trigger was reached would ensure that patients only received Oxygent therapeutically (i.e., when transfusion is clinically warranted), thereby allowing the drug to be evaluated in a manner consistent with the way blood transfusions are typically administered.

Alliance submitted this new Phase III protocol to the European Agency for the Evaluation of Medical Products (EMEA) in 2004, to seek formal scientific advice regarding development of Oxygent as an alternative to blood transfusions in elective surgery. Contrary to previous informal guidance received from regulatory authorities in key European countries (including France, the Netherlands and the UK), the EMEA indicated that if avoidance of blood transfusion were to be pursued as a primary endpoint the safety comparison would be complex, and suggested that Alliance consider an initial indication that would not require a direct comparison to allogeneic blood transfusion. Alliance requested further clarification of this opinion, which made it clear that the EMEA did not believe it was feasible at that time to conduct a study that was of sufficient size to demonstrate equivalent safety of an oxygen therapeutic with donor blood, based on the low incidence of serious adverse effects known to be associated with transfusion of allogeneic blood (e.g., death, and transmission of viral diseases caused by HIV and hepatitis contamination). Due to this regulatory opinion from the EMEA, Alliance decided that it would be necessary to pursue an alternative clinical indication for the initial commercialization of Oxygent, namely, to exploit the efficient oxygen delivery capability of Oxygent to enhance tissue oxygenation and protect organs from ischemic injury, thereby potentially decreasing postoperative complications arising from acute tissue hypoxia during elective surgery.

Since late 2002, following the setback in clinical development because of the safety findings in their Phase III CABG trial, Alliance had to downsize the company and terminate all development work on Oxygent due to lack of funding and the dissolution of a joint venture licensing agreement with Baxter Healthcare. Subsequently, throughout 2004, Alliance was actively engaged in licensing discussions with potential pharmaceutical partners willing to provide the necessary resources to complete the remaining clinical and regulatory development necessary for future commercialization of Oxygent in Europe and in Asia. In parallel, efforts continued to secure sufficient new financing that would support contract manufacturing, and the remaining clinical development needed for future commercialization of Oxygent in North America.

In addition to elective surgery, a number of other possible applications exist for Oxygent that may be pursued in the future. These include trauma resuscitation and urgent situations where donor blood is necessary for transfusion but not immediately accessible. Oxygent could provide tissues with immediate oxygenation to delay transfusion until a definitive need for blood can be established, or could provide a temporary 'oxygenation bridge' to stabilize patients until blood becomes available. Future medical indications may focus on augmentation of tumor oxygen levels to enhance sensitivity

to radiation and chemotherapy, as a treatment for reversing sickle cell crisis, as a post-dive treatment for decompression sickness, and for preservation of tissues and organs destined for transplantation.

Editor's Summary

Oxygent, like Oxyfluor, is a 'second-generation' perfluorocarbon-based emulsion. Oxygent contains 60 g of the perfluorochemical perflubron per 100 mL of emulsion, and also contains a small quantity of perflubrodec (perfluorodecyl bromide; $C_{10}F_{21}Br$), added as a stabilizer. It is emulsified with egg-yolk phospholipid (EYP). The concentration and oxygen capacity of Oxygent is approximately three times that of Fluosol and Perftoran. The formulation and manufacturing of Oxygent was optimized, and the stability of the emulsion enabled it to be stored for long periods of time. Early formulations of Oxygent induced transient inflammatory responses in animals and humans, found to be related to particle size. Subsequent formulations overcame these side effects. Phase I and II clinical studies showed both safety and efficacy, but safety concerns were raised in a pivotal Phase III cardiac surgery study in which patients who received Oxygent experienced strokes significantly more frequently than control patients. The developer of Oxygent, Alliance Pharmaceutical Corp., attributed these results to defects in the design of the clinical trials and procedures for the use of Oxygent, rather than to inherent properties of the emulsion. Nevertheless, economic pressures forced cessation of clinical development.

Acknowledgments The author would like to express his sincere appreciation to Alliance Pharmaceutical Corp. for providing him the opportunity to oversee and contribute to the development of Oxygent from 1990 to 2004. The author also specifically thanks Mr. Duane Roth, Chairman, and Dr. Simon Faithfull, former Vice President, Medical Research, for their assistance and helpful feedback during preparation of various sections of this chapter.

References

1. Batra S, Keipert PE, Bradley JD, et al. Use of a PFC-based oxygen carrier to lower the transfusion trigger in a canine model of hemodilution and surgical blood loss. Adv Exp Med Biol. 1997;411:377–81.
2. Brasile L, Del Vecchio P, Amyot K, et al. Organ preservation without extreme hypothermia using an *Oxygent*™ supplemented perfusate. Artif Cells Blood Substit Immobil Biotechnol. 1994;22(4):1463–8.
3. Braun RD, Linsenmeier RA, Goldstick TK. New perfluorocarbon emulsion improves tissue oxygenation in cat retina. J Appl Physiol. 1992;72(5):1960–8.
4. Cain SM, Curtis SE, Vallet B, Bradley WE. Resuscitation of dogs from endotoxic shock by continuous dextran infusion with and without perflubron. Adv Exp Mol Biol. 1994;345:235–42.
5. Chapman KW, Keipert PE, Graham HA. Commercial scale production of perfluorochemical emulsions. Artif Cells Blood Substit Immobil Biotechnol. 1996;24:318.
6. Daugherty WP, Levasseur JE, Sun D, et al. Perfluorocarbon emulsion improves cerebral oxygenation and mitochondrial function after fluid percussion brain injury in rats. Neurosurgery. 2004;54(5):1223–30.
7. del Balzo U, Kornbrust ES, Strnat CA, et al. Hemostasis, hemodynamic and cardiac effects of cardiopulmonary bypass with perflubron emulsion. J Invest Med. 1996;44:415A.
8. Dromsky DM, Fahlman A, Spiess BD. Treatment of decompression sickness in swine with intravenous perfluorocarbon emulsion. Aviation Space Envir Fortschr Med. 2004;75(4):301–5.
9. Evans RG, Kimler BF, Morantz RA, Batnitzky S. Lack of complications in long-term Survivors after treatment with *Fluosol* and oxygen as an adjuvant to radiation therapy for high-grade brain tumors. Int J Radiat Oncol Biol Phys. 1993;26(4):649–52.
10. Flaim SF. Pharmacokinetics and side effects of perfluorocarbon-based blood substitutes. Artif Cells Blood Substit Immobil Biotechnol. 1994;22(4):1043–54.
11. Flaim SF. Efficacy of perflubron-based emulsion as a temporary oxygen carrier. In: Rudolph AS, Rabinovici R, Feuerstein GZ, editors. Red Blood Cell Substitutes: Basic Principles and Clinical Applications. New York: Marcel Dekker, Inc.; 1998. p. 79–117.
12. Frumento RJ, Mongero L, Naka Y, Bennett-Guerrero E. Preserved gastric tonometric variables in cardiac surgical patients administered intravenous perflubron emulsion. Anesth Analg. 2002;94:809–14.
13. Gayeski TE, Connett RJ, Voter WA, et al. Whole animal lung and muscle hemodynamics and function are maintained during and after *Oxygent*™ HT infusion. Adv Exp Med Biol. 1994;361:313–8.
14. Guo G, White JA, Batjer HH. Intravenous perflubron emulsion administration improves the recovery of auditory evoked potentials after temporary brain stem ischemia in dogs. Neurosurgery. 1995;36(2):350–6.
15. Habler O, Kleen M, Hutter J, et al. IV perflubron emulsion versus autologous transfusion in severe normovolemic anemia: effects on left ventricular perfusion and function. Res Exp Med. 1998a;197:301–18.
16. Habler OP, Kleen MS, Hutter JW, et al. Hemodilution and intravenous perflubron emulsion as an alternative to blood transfusion: effects on tissue oxygenation during profound hemodilution in anesthetized dogs. Transfusion. 1998b;38:145–55.
17. Hill SE, Leone BJ, Faithfull NS, et al. Use of perflubron emulsion (AF0144) enables augmented harvesting of autologous blood and reduces allogeneic blood transfusion: a phase II study in cardiac surgery. J Cardiothorac Vasc Anesth. 2002;16(5):555–60.
18. Hogan MC, Wilford DC, Keipert PE, et al. Increased plasma O_2 solubility improves O_2 uptake of *in situ* dog muscle working maximally. J Appl Physiol. 1992;73(6):2470–5.
19. Holman WL, Spruell RD, Ferguson ER, et al. Tissue oxygenation with graded dissolved oxygen delivery during cardiopulmonary bypass. J Thorac Cardiovasc Surg. 1995;110:774–85.
20. Ingram DA, Forman MB, Murray JJ. Activation of complement by *Fluosol*® attributable to the pluronic detergent micelle structure. J Cardiovasc Pharmacol. 1993;22(3):456–61.
21. Johnson EC, Erickson BK, Podolsky A, et al. Effects of a perfluorocarbon emulsion for enhanced O_2 solubility on hemodynamics and O_2 transport in dogs. J Appl Physiol. 1995;79:1777–86.
22. Kaul DK, Liu X, Nagel RL. Ameliorating effects of fluorocarbon emulsion on sickle red blood cell-induced obstruction in an *ex vivo* vasculature. Blood. 2001;98(10):3128–31.
23. Keipert PE. Perfluorochemical emulsions – future alternatives to transfusion. In: Chang TMS, editor. Blood Substitutes: Principles,

Methods, Products and Clinical Trials. Basel: Karger; 1998. p. 127–56.
24. Keipert PE, Stehling L. The concept of augmented acute normovolemic hemodilution: using a perfluorochemical-based intravenous oxygen carrier to decrease allogeneic blood transfusion in elective surgery. Transf Alt Transf Med. 2001;3(3):11–6.
25. Keipert PE, Otto S, Flaim SF, et al. Influence of perflubron emulsion particle size on blood half-life and febrile response in rats. Artif Cells Blood Substit Immobil Biotechnol. 1994;22:1169–74.
26. Keipert PE, Batra S, Peters BP, et al. A perflubron-based oxygen carrier improves gut oxygenation in a dog model of surgical anemia. Anesthesiology. 1996a;85(3A):A219.
27. Keipert PE, Faithfull NS, Roth DJ, et al. Supporting tissue oxygenation during acute surgical bleeding using a perfluorochemical-based oxygen carrier. Adv Exp Med Biol. 1996b;308:603–9.
28. Klein DH, Jones RC, Keipert PE, et al. Intravascular behavior of perflubron emulsions. Coll Surf A: Physicochem Engr Aspects. 1994;84:89–95.
29. Klein M, Mahoney CB, Probst C, et al. Blood product use during routine open heart surgery: the impact of the centrifugal pump. Artif Organs. 2001;25(4):300–5.
30. Leese PT, Noveck RJ, Shorr JS, et al. Randomized safety studies of intravenous perflubron emulsion. I: Effects on coagulation function in healthy volunteers. Anesth Analg. 2000;91(4):804–11.
31. Manning JE, Batson DN, Payne FB, et al. Selective aortic arch perfusion during cardiac arrest: enhanced resuscitation using oxygenated perflubron emulsion, with and without aortic arch epinephrine. Ann Emerg Med. 1997;29:580–7.
32. Monk T, Winston R, Wahr J, et al. Perflubron emulsion is more effective than blood for transfusion trigger reversal. Anesth Analg. 1998a;86(2S):S142.
33. Monk TG, Winston RS, Wahr JA, et al. A prospective evaluation of the effects of perflubron emulsion on oxygen dynamics in patients undergoing moderate blood loss surgery. Anesthesiology. 1998b;89(3A):A396.
34. Noveck RJ, Shannon EJ, Leese PT, et al. Randomized safety studies of intravenous perflubron emulsion. II: Effects on immune function in healthy volunteers. Anesth Analg. 2000;91(4):812–22.
35. Padnick LB, Linsenmeier RA, Goldstick TK. Perfluorocarbon emulsion improves oxygenation of the cat primary visual cortex. J Appl Physiol. 1999;86(5):1497–504.
36. Pan W, Pintar T, Anton J, et al. Statins are associated with a reduced incidence of perioperative mortality after coronary artery bypass graft surgery. Circulation. 2004;110(11 Suppl. 1):II45–II49.
37. Paxian M, Rensing I, Geckeis K, et al. Perflubron emulsion in the treatment of prolonged hemorrhagic shock: influence on hepatocellular energy metabolism and oxygen-dependent gene expression. Anesthesiology. 2003;98:1391–9.
38. Reindorf CA, Kurantsin-Mills J, Allotey JB, Castro O. Perfluorocarbon compounds: effects on the rheological properties of sickle erythrocytes *in vitro*. Am J Hematol. 1985;19(3):229–36.
39. Riess JG. Fluorocarbon-based *in vivo* oxygen transport and delivery systems. Vox Sang. 1991;61:225–39.
40. Riess JG. Oxygen carriers ('blood substitutes') – raison d'etre, chemistry, and some physiology. Chem Rev. 2001;101:2797–919.
41. Rockwell S, Irvin CG, Kelley M, et al. Effects of hyperbaric oxygen and a perfluorooctylbromide emulsion on the radiation responses of tumors and normal tissues in rodents. Int J Radiat Oncol Biol Phys. 1992;22(1):87–93.
42. Spahn DR, von Brempt R, Theilmeier G, et al. Perflubron emulsion delays blood transfusions in orthopedic surgery. Anesthesiology. 1999;91:1195–208.
43. Spahn DR, Waschke K, Standl T, et al. Use of perflubron emulsion to decrease allogeneic blood transfusion in high-blood-loss non-cardiac surgery: results of a European Phase III study. Anesthesiology. 2002;97(6):1338–49.
44. Spiess BD, Braverman B, Woronowicz AW, et al. Protection from cerebral air emboli with perfluorocarbons in rabbits. Stroke. 1986;17:1146–9.
45. Spiess BD, McCarthy RJ, Tuman KJ, et al. Treatment of decompression sickness with a perfluorocarbon emulsion (FC-43). Undersea Biomed Res. 1988;15(1):31–7.
46. Stern SA, Dronen SC, McGoron AJ, et al. Effect of supplemental perfluorocarbon administration on hypotensive resuscitation of severe uncontrolled hemorrhage. Am J Emerg Med. 1995;13:269–75.
47. Symons JD, Sun X, Flaim SF, del Balzo U. Perflubron emulsion improves tolerance to low-flow ischemia in isolated rabbit hearts. J Cardiovasc Pharmacol. 1999;34(1):108–15.
48. Teicher BA, Holden SA, Ara G, et al. A new concentrated perfluorochemical emulsion and carbogen breathing as an adjuvant to treatment with antitumor alkylating agents. J Cancer Res Clin Oncol. 1992;118(7):509–14.
49. Teicher BA, Sotomayor EA, Dupuis NP, et al. Reduced oxygenation in a rat mammary carcinoma after chemo-or radiation therapy and reoxygenation with perflubron emulsion/carbogen breathing. J Cancer Res Clin Oncol. 1994;120(10):593–8.
50. van Rossem K, Vermarien H, Faithfull NS, et al. Effects of perflubron emulsion and 100% oxygen breathing on local tissue P_{O_2} in brain cortex of unanesthetized rabbits. Adv Exp Med Biol. 1997;411:403–9.
51. Wahr JA, Trouwborst A, Spence RK, et al. A pilot study of the effects of perflubron emulsion, AF0104, on mixed venous oxygen tension in anesthetized surgical patients. Anesth Analg. 1996;82:103–7.
52. Waschke K, Riedel M, Albrecht DM, et al. Effects of a perfluorocarbon emulsion on regional cerebral blood flow and metabolism after fluid resuscitation from hemorrhage in conscious rats. Anesth Analg. 1994;79:874–82.
53. Weers JG. A physicochemical evaluation of perfluorochemicals for oxygen transport applications. J Fluorine Chem. 1993;64:73–93.

Sanguinate: History and Clinical Evaluation of a Multimodal HBOCs

33

Bryan T. Romito, Jia W. Romito, and Abe Abuchowski

Introduction

The development of a safe, efficacious resuscitation fluid would reduce the need for allogenic erythrocyte transfusion and provide an additional therapeutic option to patients in hemorrhagic shock. Amberson et al. described the first use of a hemoglobin-based oxygen carrier (HBOC) in the 1930s when they infused a bovine hemoglobin solution into several mammalian species [1]. Since that time, several HBOCs have been developed and examined in clinical investigations with varying degrees of success. While newer compounds are improvements on their predecessors, the adverse effects of renal toxicity, nitric oxide scavenging-induced vasoconstriction, and methemoglobinemia continue to represent limitations to their widespread use [2]. A 2008 meta-analysis concluded that the use of hemoglobin-based substitutes was associated with a significantly increased risk of death and myocardial infarction [3]. Sanguinate® (PEGylated carboxyhemoglobin bovine) (Prolong Pharmaceuticals, LLC, South Plainfield, NJ, USA) represents a later generation HBOC that possesses unique properties designed to improve its efficacy and safety profile. It was developed for the treatment of anemic and ischemic hypoxia from cerebrovascular or peripheral vascular diseases and from hemoglobinopathies or vasculopathies associated with sickle cell disease (SCD) and thalassemia [4]. Although not yet approved by the US Food and Drug Administration (FDA) for standard practice, Sanguinate has been granted orphan drug status for the treatment of SCD and has been used in over 100 patients under the FDA's expanded access emergency investigational new drug (eIND) program [5, 6]. The present chapter will provide an overview of its structural and functional characteristics, performance in clinical trials, and use in other clinical settings.

Sanguinate Characteristics

Structurally, Sanguinate is a polyethylene glycol (PEG) modified form of bovine hemoglobin with a carbon monoxide (CO) moiety attached to the PEGylated hemoglobin protein [7]. PEGylated bovine hemoglobin has a higher oxygen affinity than human hemoglobin, which facilitates offloading of oxygen to the hypoxic tissues. Although Sanguinate only has a hemoglobin concentration of 4–5 g/dL, it has several novel features that make it an ideal therapeutic option for patients with impaired tissue oxygen delivery, including PEG-modification (PEGylation) to enhance the circulating life, the ability to deliver CO, and an advantageous hemoglobin-oxygen binding affinity [8] (Table 33.1). These properties are especially valuable following traumatic events.

Trauma is the leading cause of death worldwide [9]. Mortality after injury is due to "first hits", including severe organ injury, hypoxia, hypovolemia, or head trauma [10]. Upwards of 40% of trauma fatalities are due to hemorrhage and hemorrhagic shock [11]. A local inflammatory response always occurs in relation to trauma. Severe injury or multiple traumas evoke a systemic inflammatory response [12]. Sanguinate first acts as a carbon monoxide releasing molecule. Amongst the most important applications of CO in

Table 33.1 Sanguinate characteristics

Hemoglobin source	Bovine
Hemoglobin concentration	4–5 g/dL
P50	7–16 mmHg
Half-life	8–20 h
Colloid oncotic pressure	Hyperoncotic
Molecular size	109–120 kDs

B. T. Romito (✉)
Department of Anesthesiology and Pain Management, University of Texas Southwestern Medical Center, Dallas, TX, USA
e-mail: Bryan.Romito@utsouthwestern.edu

J. W. Romito
Departments of Anesthesiology and Pain Management, Neurological Surgery, and Neurology, UT Southwestern Medical Center, Dallas, TX, USA

A. Abuchowski
Prolong, Inc., South Plainfield, NJ, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_33

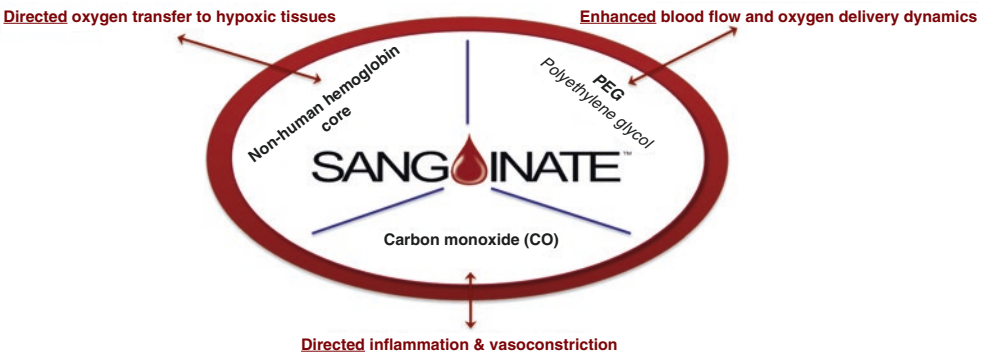


Fig. 33.1 Sanguinate properties

mammalian physiology and medicine are its vasoactive properties and the therapeutic potentials in vascular disease, anti-inflammatory effects, CO-mediated cell signaling in apoptosis and organ preservation as well as ischemia reperfusion injury and sepsis [13]. The multitude of physiological effects have been recently reviewed [14]. The multiple properties of Sanguinate may make it an ideal resuscitation fluid capable of treating a variety of conditions (Fig. 33.1).

PEGylation

The process of PEGylation involves modifying biologic molecules via covalent conjugation to PEG. Attaching PEG profoundly alters the structural and functional characteristics of the parent compound [15]. PEG itself is biologically inert, is non-toxic, and has very low immunogenicity. These properties can help improve the safety profile and effectiveness of the underlying molecule by shielding it from the native immune system. The immune response to a foreign compound may not only interfere with its efficacy but also induce a dangerous cascade of inflammatory-mediated actions in response to its detection [16]. This response is mitigated by PEGylation. Additionally, PEGylation alters the pharmacokinetics of the molecule. PEG-modification typically improves drug solubility, increases circulating half-life, and reduces proteolysis and renal excretion [15]. The reported half-life of Sanguinate ranges from 8 to 20 h [4, 17]. PEGylation increases the molecular size and colloid oncotic pressure (COP) of the parent compound. The increase in COP both helps to prevent extravasation into the interstitium and draws fluid into the intravascular space, potentially augmenting microvascular perfusion [7, 18]. Sanguinate is primarily composed of carboxylated bovine hemoglobin molecules with eight to ten 5 kilodalton (kD) PEGs attached, resulting in a total molecular weight of approximately 109–120 kDs. Although PEGylation has increased the size of Sanguinate, the particles are still much smaller than erythrocytes and thus are able to bypass obstructions and erythrocyte aggregates present in the vasculature [7]. In this way, Sanguinate can more readily traverse constricted areas of plasma that may prevent the passage of normal erythrocytes.

Multiple PEG-hemoglobins have been produced by other groups and their physiological characteristics differ. Normally, plasma hemoglobin scavenges nitric oxide (NO) produced by endothelial cells. This reduction in NO concentration prevents smooth muscle relaxation, producing local vasoconstriction with a reduction in blood flow [19]. The overall impact of PEGylated hemoglobin compounds on NO concentration and vasoactivity is variable. Like uncongested hemoglobin, PEGylated hemoglobin lowers perivascular NO because of increased scavenging. By itself, this would result in vasoconstriction and impaired blood flow [20, 21]. PEGylated hemoglobin compounds may compensate for the increased NO scavenging by also increasing the production of NO from enhanced nitrite to NO reductase activity [20, 22]. This effect would be associated with vasodilation and increased blood flow. Furthermore, excessive vasoconstriction does not typically occur because of the low PEGylated hemoglobin concentration in the circulation. PEGylation-mediated increases in COP pull fluid from the interstitial space, thus decreasing the overall concentration in the plasma by increasing volume [20]. Sanguinate differs from these PEG-hemoglobins in that it is modified with CO and displays no negative but rather positive vasoactivity.

Carbon Monoxide Delivery

Unlike other HBOCs, Sanguinate is a gas-transfer agent with unusual binding characteristics. Sanguinate has a greater affinity for oxygen than CO, which is the reverse of human hemoglobin. Upon entering the bloodstream, oxygen displaces the bound CO, which is released [8]. Because the oxygen is tightly bound, it is only transferred to tissues with a lower partial pressure of oxygen, that is, hypoxic tissue. Normoxic tissues have too high a partial pressure of oxygen to remove the oxygen from Sanguinate which makes the product highly selective and efficient.

CO is produced endogenously as a byproduct of heme oxygenase activity and is a second messenger involved in

several homeostatic functions. These pathways impact nearly every organ and regulatory system [23]. Through modification of these signaling pathways, CO can exert profound effects on the regulation of vasoactivity, inflammation, apoptosis, and cell growth [24]. As a gas, it is freely diffusible and thus passes through all membranes, bypassing receptors and transporters [25]. Its main physiologic actions are in part mediated by its activation of guanylate cyclase with resultant increase in cyclic guanosine monophosphate (cGMP) concentrations [26]. This increase in cGMP levels is associated with relaxation of vascular smooth muscle, vasodilation, and increased local blood flow. The vasodilatory effects of CO may help to mitigate elevations in blood pressure that can be associated with administration of substances with a high COP. Additionally, CO is closely interrelated with NO. CO increases the activity of nitric oxide synthase, and the NO that is produced in turn activates heme oxygenase, increasing CO generation [25]. In this way, the vasodilatory effects of NO can be potentiated by CO.

Separate from its effects on NO production, CO also may possess anti-inflammatory properties. It regulates the generation of reactive oxygen species as a result of CO-mediated inhibition of cellular cytochrome C oxidase [23]. Furthermore, it is able to reduce the production of several pro-inflammatory mediators in response to bacterial infections [25]. Exogenously-delivered CO has been shown to reduce oxidative stress and modulate ischemia-reperfusion injury. As vasoconstriction and inflammation can induce further tissue damage and exacerbate the impact of impaired oxygen delivery, a resuscitation fluid with the ability to also deliver CO would be highly effective in both improving tissue oxygenation and mitigating further injury [8].

Upon exposure to the oxygenated environment of blood, CO is rapidly displaced and Sanguinate is converted to PEG-oxyhemoglobin for normal oxygen carrying functions [7, 27]. Animal studies have shown that Sanguinate's bound CO component is released within 2 h following infusion, with the majority released with 30 min [17]. This short onset time may be especially advantageous in settings of acute, life-threatening tissue ischemia. Furthermore, the addition of CO reduces auto-oxidation of hemoglobin, decreasing the formation of methemoglobin and improving the shelf-life of the product [28].

Hemoglobin-Oxygen Binding

The hemoglobin-oxygen dissociation curve describes the relationship between the percent of hemoglobin saturated with oxygen as a function of the partial pressure of oxygen dissolved in the blood (PO_2) [29]. The curve is sigmoid in shape because of hemoglobin's unique binding properties. Specifically, the binding of one molecule of oxygen facilitates the binding of subsequent oxygen molecules. Thus, hemoglobin's affinity for oxygen increases until all four of its available binding sites are occupied [29]. As the percent saturation increases, the shape of the curve flattens as all the hemoglobin molecules approach complete saturation. The PO_2 at which hemoglobin is 50% saturated with oxygen is termed the P50 and is normally ~27 mmHg. The relationship between hemoglobin-oxygen saturation and PO_2 is dynamic and influenced by several physiologic factors. To adapt to these changes, the curve can be shifted to the right or left, denoting changes in the hemoglobin affinity for oxygen [29]. Specifically, rightward shifts of the curve indicate a decreased hemoglobin affinity for oxygen (i.e., a lower saturation for a given PO_2) and are frequently associated with hypercarbia, lower pH, and higher temperature. These shifts are associated with an increase in P50. Conversely, leftward shifts indicate an increased hemoglobin affinity for oxygen (i.e., a greater saturation for a given PO_2) and are frequently associated with hypocarbia, higher pH, and lower temperature [29]. These shifts are associated with a reduction in P50.

Part of Sanguinate's ability to actively transfer oxygen to hypoxic tissues is mediated by its unique hemoglobin-oxygen binding affinity. Sanguinate has an average P50 of 7–16 mmHg. Because this value is between that of normal erythrocytes (~27 mmHg) and ischemic tissues (<5 mmHg), Sanguinate acts as a conduit to transfer oxygen from erythrocytes to ischemic tissue [17]. This characteristic may be especially beneficial in the microcirculatory environment characterized by diverging vessel bifurcations. Microvessel networks are largely heterogenous and associated with variations in hematocrit, velocity, flow rate, and wall shear stress [30]. Because of hydrodynamic forces, erythrocytes flowing near blood vessel walls tend to migrate away from the walls to the center of the flow stream, forming a cell-free layer (the Fåhraeus effect) [17, 30]. Because oxygen dissolves poorly in this cell-free layer, a small molecule dissolved in this cell free layer that can transfer oxygen to ischemic tissues within microvessel networks would be a valuable treatment option.

Clinical Trials Using Sanguinate

Only results from three phase I trials using Sanguinate have been published [4, 17, 31]. A phase II trial of Sanguinate for the treatment of vaso-occlusive crisis in adult patients with SCD has been completed, but the results have not yet been published. Misra et al. published the results of the first phase I trial using Sanguinate in 2014 [17]. In this trial, researchers performed a single-center, single-blind, placebo-controlled, evaluation of the safety and pharmacokinetics of Sanguinate in 24 healthy adult volunteers. All subjects were between the ages of 18 and 45 years, had a body mass index of $\geq$20 and $\leq$30 kg/m^2, were in good general health, and had

no clinically significant illnesses. Three doses of Sanguinate (80 mg/kg, 120 mg/kg, and 160 mg/kg) were administered by intravenous infusion over two hours to three respective cohorts. Each cohort of eight subjects included six who received Sanguinate and two who received a control. Subjects were monitored as inpatients for at least 48 h following administration of either Sanguinate or control, and serial blood samples for pharmacokinetic analysis were collected at regular intervals. Plasma concentrations of Sanguinate were observed 30 min after the start of the infusion. The highest mean plasma concentration in all dose groups occurred at the 2-h point, corresponding to the end of the infusion. All Sanguinate plasma concentrations were below the limit of detection 96 h after the end of the infusion [17].

Sanguinate was overall well-tolerated in this study. There were no serious adverse events identified, and the most commonly reported adverse events (lethargy and dizziness) were mild in severity and resolved by the conclusion of the study. A consistent, benign, dose-dependent reduction in serum haptoglobin was observed in all subjects treated with Sanguinate. This result was explained by the binding of haptoglobin to Sanguinate followed by subsequent elimination from the plasma. There was a trend toward increased both systolic and diastolic blood pressure in subjects who received Sanguinate. These changes were transient and resolved by 72 h after the end of the infusion. Sanguinate-induced hypertension was not dose-dependent, suggesting that it was caused by expansion of the plasma mediated by the compound's high COP and not vasoconstriction. The hypertensive responses were not associated with clinically significant changes in electrocardiogram (ECG), echocardiogram, or laboratory findings [17].

In a follow-up evaluation, Misra et al. conducted a phase Ib, open label, randomized safety study of Sanguinate in 24 adult patients with sickle cell anemia (homozygotes for hemoglobin SS) [4]. The study was performed in four medical centers across Colombia and Panama. Patients did not have to be taking hydroxyurea, but if so, they had to be on a stable dose and be able to discontinue the medication for 7 days prior to randomization. All patients had a baseline hemoglobin concentration between 6 and 10 g/dL. All patients were randomized in a 2:1 ratio to receive, unblinded, either a single 2-h intravenous infusion of Sanguinate or a standard dose of hydroxyurea. Eight patients received 160 mg/kg of Sanguinate, eight patients received 320 mg/kg of Sanguinate, and eight patients received 15 mg/kg of hydroxyurea. A 7-day safety assessment was performed, and patients remained in the study center for at least 48 h after the start of dosing to monitor vital sign activity, collect laboratory specimens, and assess for signs or symptoms of adverse events [4].

Of the 24 patients, 15 patients received their assigned Sanguinate and seven patients received their assigned hydroxyurea. Two patients left the study prior to receiving their assigned medications. Sanguinate displayed dose-dependent pharmacokinetics as the mean peak plasma concentration was greater in the 320 mg/kg dose group. Irrespective of the dose, the maximum plasma concentration occurred at the completion of the infusion. In this study, there were 44 adverse events reported in the Sanguinate group versus seven adverse events reported in the hydroxyurea group. The most commonly reported type of adverse event was musculoskeletal and connective tissue disorder-related, including arthralgia. Sanguinate caused transient, dose-independent increases in systolic and diastolic blood pressures, again thought to be related to its COP-mediated plasma expansion properties. Compared to patients receiving hydroxyurea, Sanguinate administration resulted in decreased levels of serum direct (conjugated) bilirubin and increased levels of urinary erythrocytes and protein. It was believed that the hematuria may have been the result of Sanguinate's elevated COP causing an increase in forced glomerular filtration [4].

There were transient (lasting a few hours or days) but substantial increases in troponin I levels in 3 of the 15 patients who received Sanguinate. All patients with troponin I elevations received 320 mg/kg of Sanguinate. None of the troponin I increases were associated with any clinically identified or patient reported adverse events. One of the three patients who experienced an increased troponin I level also had an increase in their tricuspid regurgitation velocity (TRV) observed on echocardiography lasting several days. This was labeled as a sign of moderate pulmonary hypertension, but subsequent angiography did not reveal any associated pulmonary hypertension symptoms. Furthermore, this increase in TRV was not associated with any patient reported or clinically identified adverse effects. The clinical significance of these findings is not clear. Because of the small study size, it was not possible to calculate statistically significant differences in safety-related effects of Sanguinate in this population. Similar to the initial phase I trial, administration of Sanguinate produced a transient, dose-independent increase in arterial blood pressure because of its high COP. It is possible that this colloid oncotic-mediated effect may have also increased pulmonary arterial pressure and subsequently TRV in the patient described above [4].

Abu Jawdeh et al. performed a Phase Ib, open-label, single arm study to assess the safety, pharmacokinetics, and impact on humoral sensitization of Sanguinate in patients with end-stage renal disease [31]. Ten eligible subjects were planned to receive three weekly infusions of Sanguinate (320 mg/kg) over consecutive weeks. Inclusion

criteria included subjects stable on three times per week hemodialysis for ≥2 months, with a negative serum pregnancy test, and a hemoglobin >7.5 g/dL. Subjects were excluded if they had received a blood product transfusion within the previous 90 days from screening or had ECG findings suggestive of acute coronary syndrome, decompensated heart failure, arrythmias associated with hemodynamic instability, or third-degree atrioventricular block. Because of safety data reported from Misra et al.'s 2017 Phase Ib trial, subjects with echocardiographic estimates of tricuspid regurgitation jet velocity >2.8 m/s were also excluded from the study [31].

Researchers only enrolled five out of the 10 total expected subjects. The study was terminated after subject five because of troponin I elevations observed in two subjects. Overall, only four subjects completed the study, and subject five withdrew consent after receiving only one Sanguinate infusion. One of the two subjects with a troponin I elevation also experienced nonspecific ECG changes and chest pain, ultimately being diagnosed with a non-ST elevation myocardial infarction (NSTEMI). A coronary angiogram was performed for further evaluation and revealed an 80% right coronary artery lesion that underwent stenting. Of note, the patient later admitted to actively using cocaine following their NSTEMI diagnosis. The other subject who developed a troponin I elevation did not report chest pain nor have associated ECG changes or coronary angiography abnormalities. Calculated panel reactive antibody values, quantitative measures of sensitization for transplant candidates, were not increased in any of the five subjects following Sanguinate infusion. Similarly, no subjects developed new class I or II anti-HLA antibodies [31].

The immunological findings reported by this study were expected as Sanguinate is acellular and PEGylated, which safeguards it from the native immune system response. This property could make it a valuable treatment adjunct for patients awaiting transplantation. Unexpectedly, all post-dialysis Sanguinate concentrations were reduced by 30% as compared to pre-dialysis concentrations, implying some degree of clearance. Sanguinate's molecular weight should make it too large to be eliminated by dialysis. Like the previously published phase Ib study, subjects in this study experienced elevations in troponin I values. Authors postulated that demand ischemia resulting from transient Sanguinate-induced ventricular stretch may have contributed to the troponin I elevation. The study's results may have been confounded by active cocaine use reported by the subject who was diagnosed with an NSTEMI. Nevertheless, the results of these studies indicate a possible risk of myocardial injury with the use of Sanguinate. Additional larger studies are needed to closely evaluate Sanguinate's safety profile [31].

Case Reports and Other Trials

Although initially designed for the treatment of acute exacerbations of sickle cell anemia, Sanguinate has been used in several other clinical scenarios. There have been multiple case reports describing the use of Sanguinate in anemic Jehovah's Witness patients who chose to abstain from blood transfusions for religious reasons. McConachie et al. published a case series in which Sanguinate was administered to two Jehovah's Witness patients with severe, life-threatening anemia [32]. The first patient received a single 2-h infusion of Sanguinate (approximately 210 mg/kg) in addition to daily erythropoietin, ferric gluconate, ascorbic acid, folate, and vitamin B12. The patient's reported past medical history was notable for hypertension, congestive heart failure, coronary artery disease, stroke, and paroxysmal atrial fibrillation. During the infusion, the patient experienced an increase in their systolic and diastolic blood pressure, which normalized 2 h later. This patient also had an elevation in their troponin level, which was attributed to acute anemia-induced demand ischemia. Five days later, the troponin level decreased below the lower limit of detection. The patient was eventually discharged from the hospital. The second patient described received five total infusions of Sanguinate (approximately 357 mg/kg) once daily for 5 days, in addition to daily erythropoietin, ferric gluconate, folate and vitamin B12. The patient's reported past medical history was notable for drug abuse, hypertension, chronic kidney disease, seizure disorder, ischemic cardiomyopathy requiring implantation of a percutaneous ventricular assist device, and coronary artery disease with recent NSTEMI. Troponin levels were drawn 30 min following the first infusion and were not elevated. The patient ultimately died from multi-organ system failure. Despite being at very high risk for cardiovascular complications, no adverse effects from Sanguinate were identified. Overall, this small case series illustrates some of the potential benefits of Sanguinate in anemic patients in whom blood transfusion is contraindicated [32].

Bachert et al. described the administration of Sanguinate to a severely anemic Jehovah's Witness patient with no known medical history whose hemoglobin had fallen as low as 2.9 g/dL [28]. The patient received two doses of Sanguinate infusion (20,000 mg per dose, patient weight not reported) 6 h apart in addition to daily darbepoetin, iron sucrose, folic acid, and vitamin B12. He reportedly refused additional doses of Sanguinate because of rapid improvement in his symptoms. His serum demonstrated a red discoloration following administration of one dose of the product, which may impact colorimetric laboratory analysis. The patient was ultimately discharged from the hospital without complications or adverse reactions to the product, although it was not reported which laboratory studies were ordered for further evaluation.

Similarly, Brotman et al. reported a case of Sanguinate administration to a Jehovah's Witness patient with symptomatic anemia (confusion and lethargy) who declined transfusion of blood products [33]. The patient's hemoglobin dropped to 4.5 g/dL in the setting of perioperative bleeding following a cystoprostatectomy and nephrectomy. The patient's medical history was significant for urothelial carcinoma, atrial fibrillation, and coronary artery disease. Postoperatively, he was persistently hypotensive and required a phenylephrine infusion to support his hemodynamics. The patient received Sanguinate on the evening of postoperative day two, and his mental and hemodynamic status began to improve by the following day. Although the dosing regimen was not described, Sanguinate was well-tolerated with no adverse effects reported. The patient was ultimately discharged from the hospital in good condition.

Holzner et al. described the use of Sanguinate in an anemic Jehovah's Witness patient who underwent an orthotopic liver transplant [34]. The patient received a 2-h infusion of 20,000 mg of Sanguinate 60 min prior to the anhepatic phase of their transplantation. The patient's weight was not provided, so the mg/kg dose could not be calculated. Fifteen minutes following administration of Sanguinate, the patient's cerebral oxygen saturation increased considerably despite ongoing, worsening anemia with stable hemodynamic measurements. No adverse effects were associated with Sanguinate administration, and the patient was ultimately discharged from the hospital in good condition. This report illustrates that Sanguinate may be a useful treatment adjunct in anemic patients who abstain from blood transfusion but require strict maintenance of their cerebral oxygen saturation.

Thenuwara et al. reported the use of Sanguinate in a Jehovah's Witness patient with life-threatening anemia following postpartum hemorrhage [35]. The patient's hemoglobin decreased to 3 g/dL, and she required deep sedation, mechanical ventilation, and neuromuscular paralysis to decrease her work of breathing and metabolic rate of oxygen consumption along with vasopressors to support her hemodynamics. Additionally, the patient received erythropoietin, iron sucrose, cyanocobalamin, and hyperbaric oxygen therapy with no substantial improvement in clinical status. The patient received three doses of Sanguinate (each approximately 164 mg/kg) over the next 2 days with subsequent improvement in her hemodynamics. Vasopressors were able to be weaned off, and her metabolic acidosis and lactic acid elevation resolved. Approximately 48 h after receiving Sanguinate, the patient was found to be obtunded but recovered spontaneously the following day. The etiology of the patient's neurologic change was not clearly elucidated; however, the authors suggest that it may have been a result of Sanguinate administration. She was ultimately extubated, transferred out of the intensive care unit, and discharged from the hospital without apparent neurological deficits.

Sam et al. described the use of Sanguinate in a Jehovah's Witness patient with thrombotic thrombocytopenic purpura (TTP) who declined therapeutic plasma exchange [36]. The patient was treated with high dose steroids, folate, erythropoietin, albumin exchange, rituximab, and vincristine without improvement in their symptoms. The patient received a daily infusion of 20,000 mg of Sanguinate for 4 days with a subsequent improvement in platelet count and hemoglobin concentration. The patient experienced paresthesia of the right face and arm 1 h after the first Sanguinate infusion that resolved spontaneously. The patient also had a mild, transient elevation in their cardiac troponin value after receiving Sanguinate. Further workup with both an ECG and echocardiogram were unremarkable, and the patient was discharged from the hospital in good condition. This case supports the use of Sanguinate to augment oxygen delivery in patients with TTP who are unable to undergo plasma exchange.

DeSimone et al. reported the use Sanguinate in a Jehovah's Witness patient with an acute upper gastrointestinal bleed and hemorrhagic shock [37]. The patient's hemoglobin decreased to 3.1 g/dL, and she required resuscitation with intravenous fluids and vasopressors to support her hemodynamics along with intubation for progressive encephalopathy. The patient received six separate 20,000 mg infusions of Sanguinate over 7 days in addition to iron supplementation, folate, vitamin B12, and darbepoetin alfa infections. The patient's shock, encephalopathy, vasopressor requirement, and metabolic acidosis all improved following Sanguinate administration. The improvement in clinical status facilitated performance of additional procedures to identify the source of bleeding and ultimately achieve hemostasis. There were no adverse events related to the administration of Sanguinate.

Sanguinate may be able to provide a benefit in patients with cerebral ischemia. Dhar et al. performed a safety and proof-of-concept study evaluating the impact of Sanguinate in 12 adult patients with aneurysmal subarachnoid hemorrhage (SAH) with or at risk for delayed cerebral ischemia (DCI) [38]. All patients were deemed at risk for DCI based on poor clinical grade, high subarachnoid blood burden, presence of angiographic vasospasm, or clinical signs of DCI. Patients were excluded if they had pulmonary hypertension, congestive heart failure, recent myocardial infarction, renal insufficiency, or chronic liver disease. In addition to receiving nimodipine and intravenous fluids to maintain euvolemia, three escalating dose of Sanguinate (160 mg/kg, 240 mg/kg, and 320 mg/kg) were administrated by 2-h intravenous infusion to three cohorts of four patients. Positron emission tomography imaging was performed at baseline, immediately after Sanguinate administration, and at 24-h after the baseline study to evaluate for improvements in cere-

bral blood flow and restoration of flow-metabolism balance, assessed by oxygen extraction fraction. All patients underwent screening cerebral angiography approximately 7 days after SAH or earlier if symptoms concerning for DCI manifested [38].

Patients were followed for adverse events daily until discharge from the intensive care unit. Sanguinate administration was associated with an approximate 10 mmHg increase in mean arterial pressure during and immediately after infusion, although patients' blood pressures returned to baseline by 24 h. Similar to other studies, this hypertensive effect was not dose-related, with a similar increase in blood pressure reported in all three cohorts. The degree of blood pressure elevation did not exceed the safety threshold (increase in 20% from baseline) to trigger premature drug discontinuation in any case. This hypertensive effect allowed all three of the patients who were receiving vasopressors to be weaned off these agents while maintaining their target blood pressure without any associated neurologic deterioration. Sanguinate administration was associated with a statistically significant increase in carboxyhemoglobin levels. This increase was significant at all doses but larger with higher doses. Like the hypertensive effect, the carboxyhemoglobin level elevation was transient and returned to baseline by 24 h. The levels obtained immediately after infusion completion mirrored those routinely seen in smokers. No respiratory or cardiovascular complications were reported with Sanguinate infusion [38].

When combining all doses, there was no change in global cerebral blood flow following infusion of Sanguinate, although there was a significant improvement in patients within the highest dose (320 mg/kg) cohort. Global assessments of oxygen extraction fraction and cerebral metabolic rate of oxygen were unchanged at all time points. Sanguinate infusion was associated with a statistically significant improvement in regional cerebral blood flow, but this effect was not sustained at 24 h. These results suggest that repeat dosing would likely be necessary. Furthermore, there was a concomitant reduction in oxygen extraction fraction in vulnerable regions, suggesting that Sanguinate improved supply and demand matching in high risk brain regions. Although the exact mechanism is unknown, this study's results may have been explained by cerebral vasodilation mediated by the dose-dependent release of CO into the cerebral circulation following Sanguinate administration. Alternatively, Sanguinate's increase in regional cerebral blood flow may have been enhanced by hemodilution arising from its high COP effects. Aside from its impact on regional blood flow, from a safety perspective, this study's results are noteworthy as no adverse effects were identified when high dose Sanguinate was administered to a population of critically ill patients. Although patients with pulmonary hypertension and recent myocardial infarction were not included in the study and troponin I values were not measured, the results are encouraging. Additional larger studies are needed to better define its safety profile and therapeutic role in the setting of cerebral ischemia [38].

Sanguinate may have a role in augmenting tissue oxygen delivery during cardiac surgery using cardiopulmonary bypass (CPB) with intraoperative normovolemic hemodilution. This practice involves the removal of whole blood with simultaneous replacement of the harvested blood with a replacement fluid to maintain normovolemia. During or shortly after surgical blood loss, the harvested blood is then re-transfused to restore normal levels of erythrocytes, platelets, and clotting factors [6]. The addition of a HBOC to the replacement fluid during hemodilution would reduce the need for allogenic erythrocyte transfusion and provide an additional means to improve tissue oxygen delivery. This technique would offer another therapeutic option for high risk patients and be especially useful during times of blood shortage or in patients where blood transfusion is contraindicated. In a small study simulating normovolemic hemodilution with CPB, the addition of Sanguinate did not affect the performance of the CPB circuit nor alter the flow or oxygenation characteristics of the system [6]. While the impact of Sanguinate on normovolemic hemodilution and CPB requires further evaluation in large trials, the results of this study highlight another potential setting for its use.

Finally, preliminary studies indicate that the anti-inflammatory activity of Sanguinate may be useful in the treatment of cystic fibrosis (CF) and sepsis. Inflammation plays a critical role in CF lung pathology and disease progression making it an important therapeutic target [39]. The CF airway contains large numbers of neutrophils and increased concentrations of pro-inflammatory mediators that promote inflammatory cell signaling in response to noxious stimuli. The non-resolving hyper-inflammation in CF lungs is attributed to an impairment of several signaling pathways associated with resolution of the inflammatory response, including the hemeoxygenase-1/carbon monoxide (HO-1/CO) pathway [40]. HO-1 degrades heme groups, producing anti-inflammatory mediators, such as CO, which help to resolve inflammation and re-establish cellular homeostasis.

Importantly CO plays key roles in mediating protection against lung inflammation and oxidant-mediated lung injury. As such, the HO-1 pathway is an attractive target for disrupting the hyper-inflammatory response in CF [41]. The effects of Sanguinate were tested in both wild type and CF mice. The mice were pre-treated intravenously with a single clinically relevant dose of Sanguinate (320 mg/kg) or vehicle alone then exposed to 3 nebulizations with 12.5 mg *Pseudomonas aeruginosa*-lipopolysaccharide (PA-LPS) every day for 3 days. A single dose of Sanguinate was sufficient to induce HO-1 expression in lung tissues of CF mice in response to PA-LPS. Moreover, pretreatment with

Sanguinate reduced inflammation in CF mice as demonstrated by a lower number of neutrophils and a reduced expression of pro-inflammatory cytokines in CF lung tissues when compared to vehicle treated controls. Sanguinate treatment stimulated the HO-1/CO pathway, which mediates resolution of the inflammatory response and promotes host defense against *Pseudomonas aeruginosa* [41].

Sepsis is a complex, evolving heterogeneous disease process initiated by progression of an underlying severe infection with an overwhelming host systemic inflammatory response. Early diagnosis and treatment are essential for reducing morbidity/mortality in sepsis [42]. Delayed treatment results in proliferation of both bacterial counts and circulating immune cell numbers which may promote a "cytokine storm" that rapidly progresses to severe septic shock, multi-organ failure, and potentially, death [43]. Sanguinate has the potential to impact three events in the pathology of sepsis: capillary leak/hypovolemia, hyper-acute inflammatory response, and poor tissue oxygenation.

Mesenchymal stromal cells (MSCs) have been investigated as a cell-based therapy for a number of disease processes, with promising results in animal models of systemic inflammation and sepsis. Studies to determine ways to further improve the therapeutic potential of MSCs involved preconditioning of these cells with CO ex vivo to promote further therapeutic benefit when cells are administered in vivo after the onset of polymicrobial sepsis in mice [44]. The study demonstrated that preconditioning of MSCs with CO gas improved MSC function and therapeutic efficacy during sepsis. The increased beneficial response of MSCs exposed to CO occurred in part through the interaction of MSCs with neutrophils to promote bacterial phagocytosis, resolution of inflammation, and increased survival.

Sanguinate may improve both skeletal microcirculatory flow and renal cortical microcirculatory partial oxygen pressure ($C\mu PO_2$) and subsequently limit endotoxemia-induced acute kidney injury [45]. Studies were performed in anesthetized, ventilated Wistar albino rats ($n = 44$) that underwent endotoxemic shock. Rats were randomly assigned to the following five groups: (*1*) unresuscitated lipopolysaccharide (LPS), (*2*) LPS + Ringer's acetate (RA), (*3*) LPS + RA + 0.5 μg/kg/min norepinephrine (NE), (*4*) LPS + RA + 320 mg/kg Sanguinate, and (*5*) LPS + RA + Sanguinate + NE. $C\mu PO_2$ was higher in the LPS + RA + Sanguinate-resuscitated group, and the number of nonflowing, intermittent, or sluggish capillaries was smaller in groups infused with Sanguinate compared with RA alone, while the number of normally perfused vessels was greater. Sanguinate enhanced $C\mu PO_2$ while restoring skeletal muscle microcirculatory flow in previously nonflowing capillaries.

Conclusion

Sanguinate is a novel multimodal resuscitation fluid that possesses several advantages over older generations of HBOCs. Its therapeutic characteristics allow for the control of inflammation while providing effective delivery of oxygen to hypoxic tissue with low oxygen tension. It does not elicit a response from the native immune system, and its CO moiety may promote vasodilation, reduce inflammation, and mitigate ischemia-reperfusion injury. Sanguinate has demonstrated a clinical benefit in several scenarios characterized by inflammation and poor tissue oxygenation. While data from clinical trials suggest there is a possible risk of myocardial injury with the use of Sanguinate, additional studies are needed to better define this association.

Key Points

- Sanguinate is a unique polyethylene glycol modified form of bovine hemoglobin that represents the latest generation of hemoglobin-based oxygen carriers.
- It has the ability to deliver carbon monoxide, has an advantageous hemoglobin-oxygen binding affinity, and has undergone PEGylation to enhance its circulating life.
- Sanguinate possesses novel anti-inflammatory, anti-vasoconstrictive, and plasma expansion properties.
- Its characteristics allow for effective oxygen delivery to hypoxic tissues with low oxygen tension and mitigation of inflammation, making it an ideal resuscitation fluid in situations where blood is ineffective.
- Sanguinate has shown a clinical benefit in several scenarios and has been used under emergency circumstances in over 100 patients with severe anemia and impaired oxygen delivery.

References

1. Amberson WR, Flexner J, Steggerda FR, Mulder AG, Tendler MJ, Pankratz DS, et al. On the use of ringer-locke solutions containing hemoglobin as a substitute for normal blood in mammals. J Cell Comp Physiol. 1934;5(3):359–82.
2. Jahr JS, Guinn NR, Lowery DR, Shore-Lesserson L, Shander A. Blood substitutes and oxygen therapeutics: a review. Anesth Analg. 2021;132(1):119–29.
3. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA. 2008;299(19):2304–12.
4. Misra H, Bainbridge J, Berryman J, Abuchowski A, Galvez KM, Uribe LF, et al. A Phase Ib open label, randomized, safety study of Sanguinate™ in patients with sickle cell anemia. Rev Bras Hematol Hemoter. 2017;39(1):20–7.
5. Khan F, Singh K, Friedman MT. Artificial blood: the history and current perspectives of blood substitutes. Discoveries (Craiova). 2020;8(1):e104.

6. Romito BT, McBroom MM, Bryant D, Gamez J, Merchant A, Hill SE. The effect of Sanguinate(®) (PEGylated carboxyhemoglobin bovine) on cardiopulmonary bypass functionality using a bovine whole blood model of normovolemic hemodilution. Perfusion. 2020;35(1):19–25.
7. Nugent WH, Jubin R, Buontempo PJ, Kazo F, Song BK. Microvascular and systemic responses to novel PEGylated carboxyhaemoglobin-based oxygen carrier in a rat model of vaso-occlusive crisis. Artif Cells Nanomed Biotechnol. 2019;47(1):95–103.
8. Abuchowski A. Sanguinate (PEGylated Carboxyhemoglobin Bovine): mechanism of action and clinical update. Artif Organs. 2017;41(4):346–50.
9. Bartolotti P, Faure E, KIpnis E. Inflammasomes in tissue damages and immune disorders after trauma. Front Immunol. 2018;9:1900.
10. Lenz A, Franklin GA, Cheadle WG. Systemic inflammation after trauma. Injury. 2007;38(12):1336–45.
11. D'Angelo MR, Dutton RP. Management of trauma-induced coagulopathy: trends and practices. AANA J. 2010;78(1):35–40.
12. Talukder AR, Quader F, Momen MA. Pathophysiological reaction of the body to trauma: a review update. J Sci Found. 2015;13(1):15–20.
13. Davidge KS, Motterlini R, Mann BE, Wilson JL, Poole RK. Carbon monoxide in biology and microbiology: surprising roles for the "Detroit perfume". Adv Microb Physiol. 2009;56:85–167.
14. Ismailova A, Kuter D, Bohle DS, Butler IS. An overview of the potential therapeutic applications of CO-releasing molecules. Bioinorg Chem Appl. 2018;2018:8547364.
15. Veronese FM, Mero A. The impact of PEGylation on biological therapies. BioDrugs. 2008;22(5):315–29.
16. De Groot AS, Terry F, Cousens L, Martin W. Beyond humanization and de-immunization: tolerization as a method for reducing the immunogenicity of biologics. Expert Rev Clin Pharmacol. 2013;6(6):651–62.
17. Misra H, Lickliter J, Kazo F, Abuchowski A. PEGylated carboxyhemoglobin bovine (Sanguinate): results of a phase I clinical trial. Artif Organs. 2014;38(8):702–7.
18. Pasut G, Veronese FM. State of the art in PEGylation: the great versatility achieved after forty years of research. J Control Release. 2012;161(2):461–72.
19. Cabrales P. Examining and mitigating acellular hemoglobin vasoactivity. Antioxid Redox Signal. 2013;18(17):2329–41.
20. Cabrales P, Intaglietta M. Blood substitutes: evolution from noncarrying to oxygen- and gas-carrying fluids. ASAIO J. 2013;59(4):337–54.
21. Tsai AG, Cabrales P, Manjula BN, Acharya SA, Winslow RM, Intaglietta M. Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free hemoglobin oxygen carriers. Blood. 2006;108(10):3603–10.
22. Lui FE, Dong P, Kluger R. Polyethylene glycol conjugation enhances the nitrite reductase activity of native and cross-linked hemoglobin. Biochemistry. 2008;47(40):10773–80.
23. Levitt DG, Levitt MD. Carbon monoxide: a critical quantitative analysis and review of the extent and limitations of its second messenger function. Clin Pharmacol. 2015;7:37–56.
24. Hess DR. Inhaled carbon monoxide: from toxin to therapy. Respir Care. 2017;62(10):1333–42.
25. Motterlini R, Otterbein LE. The therapeutic potential of carbon monoxide. Nat Rev Drug Discov. 2010;9(9):728–43.
26. Deng X, Yasuda H, Sasaki T, Yamaya M. Low-dose carbon monoxide inhibits rhinovirus replication in human alveolar and airway epithelial cells. Tohoku J Exp Med. 2019;247(4):215–22.
27. Nugent WH, Sheppard FR, Dubick MA, Cestero RF, Darlington DN, Jubin R, et al. Microvascular and systemic impact of resuscitation with PEGylated carboxyhemoglobin-based oxygen carrier or hetastarch in a rat model of transient hemorrhagic shock. Shock. 2020;53(4):493–502.
28. Bachert SE, Dogra P, Boral LI. Alternatives to transfusion. Am J Clin Pathol. 2020;153(3):287–93.
29. Collins JA, Rudenski A, Gibson J, Howard L, O'Driscoll R. Relating oxygen partial pressure, saturation and content: the haemoglobin-oxygen dissociation curve. Breathe (Sheff). 2015;11(3):194–201.
30. Secomb TW. Hemodynamics. Compr Physiol. 2016;6(2):975–1003.
31. Abu Jawdeh BG, Woodle ES, Leino AD, Brailey P, Tremblay S, Dorst T, et al. A phase Ib, open-label, single arm study to assess the safety, pharmacokinetics, and impact on humoral sensitization of Sanguinate infusion in patients with end-stage renal disease. Clin Transplant. 2018;32(1):e13155.
32. McConachie S, Wahby K, Almadrahi Z, Wilhelm S. Early experiences with PEGylated carboxyhemoglobin bovine in anemic Jehovah's Witnesses: a case series and review of the literature. J Pharm Pract. 2020;33(3):372–7.
33. Brotman I, Kocher M, McHugh S. Bovine hemoglobin-based oxygen carrier treatment in a severely anemic Jehovah's Witness patient after cystoprostatectomy and nephrectomy: a case report. A A Pract. 2019;12(7):243–5.
34. Holzner ML, DeMaria S, Haydel B, Smith N, Flaherty D, Florman S. Pegylated bovine carboxyhemoglobin (Sanguinate) in a Jehovah's witness undergoing liver transplant: a case report. Transplant Proc. 2018;50(10):4012–4.
35. Thenuwara K, Thomas J, Ibsen M, Ituk U, Choi K, Nickel E, et al. Use of hyperbaric oxygen therapy and PEGylated carboxyhemoglobin bovine in a Jehovah's Witness with life-threatening anemia following postpartum hemorrhage. Int J Obstet Anesth. 2017;29:73–80.
36. Sam C, Desai P, Laber D, Patel A, Visweshwar N, Jaglal M. Pegylated bovine carboxyhaemoglobin utilisation in a thrombotic thrombocytopenic purpura patient. Transfus Med. 2017;27(4):300–2.
37. DeSimone RA, Berlin DA, Avecilla ST, Goss CA. Investigational use of PEGylated carboxyhemoglobin bovine in a Jehovah's Witness with hemorrhagic shock. Transfusion. 2018;58(10):2297–300.
38. Dhar R, Misra H, Diringer MN. Sanguinate™ (PEGylated carboxyhemoglobin bovine) improves cerebral blood flow to vulnerable brain regions at risk of delayed cerebral ischemia after subarachnoid hemorrhage. Neurocrit Care. 2017;27(3):341–9.
39. Roesch EA, Nichols DP, Chmiel JF. Inflammation in cystic fibrosis: an update. Pediatr Pulmonol. 2018;53(S3):S30–s50.
40. Di Pietro C, Öz HH, Murray TS, Bruscia EM. Targeting the Heme Oxygenase 1/Carbon Monoxide pathway to resolve lung hyperinflammation and restore a regulated immune response in cystic fibrosis. Front Pharmacol. 2020;11:1059.
41. Di Pietro C, Öz HH, Martis V, Zhang P, Jubin R, Abuchowski A, et al. Targeting the HO-1/CO Pathway with Sanguinate: toward development of a novel anti-inflammatory therapy for cystic fibrosis (CF) lung disease. American Thoracic Society 2019 International Conference, Dallas, 2019. p. A6187.
42. Paoli CJ, Reynolds MA, Sinha M, Gitlin M, Crouser E. Epidemiology and costs of sepsis in the United States-an analysis based on timing of diagnosis and severity level. Crit Care Med. 2018;46(12):1889–97.
43. Chousterman BG, Swirski FK, Weber GF. Cytokine storm and sepsis disease pathogenesis. Semin Immunopathol. 2017;39(5):517–28.
44. Tsoyi K, Hall SR, Dalli J, Colas RA, Ghanta S, Ith B, et al. Carbon monoxide improves efficacy of mesenchymal stromal cells during sepsis by production of specialized proresolving lipid mediators. Crit Care Med. 2016;44(12):e1236–e45.
45. Guerci P, Ergin B, Kandil A, Ince Y, Heeman P, Hilty MP, et al. Resuscitation with PEGylated carboxyhemoglobin preserves renal cortical oxygenation and improves skeletal muscle microcirculatory flow during endotoxemia. Am J Physiol Renal Physiol. 2020;318(5):F1271–F83.

M101, the Hemoglobin from the Sea: History and Therapeutic Perspectives

34

Franck Zal, Eric Delpy, and Jonathan S. Jahr

Introduction

The concept of a blood substitute/oxygen carrier, such as those produced by Hemarina, are nature based; as with societal issues, many have nature-based solutions. Biomimicry may improve the efficiency of healthcare, taking advantage of solutions vetted by nature during millions of years of evolution. Evolution provides an understanding of what works best and what may not. Leonardo Da Vinci's statement: "take your lessons in nature, this is our future", is the underlying concept of Hemarina's development and how a marine worm found on the beach at low tides may be such a natural evolution to provide impetus towards creating a novel and unique oxygen therapeutic. By understanding, sharing, and translating the properties of this worm's hemoglobin to the world of physiology and medicine, a new therapeutic era has been created.

Sea organisms are intriguing animals, with relatively little known about their biology. Given that the ocean covers approximately 71% of the earth's surface and represents 90% of the earth's biosphere, the amount known about these organisms is miniscule compared to vertebrates and especially primates. The intertidal area is a critical ecosystem, in that the transition between the marine world and the terrestrial world, defines aquatic and land life. All forms of life that survived on land transitioned through this area during evolution.

Following is a brief history of the scientist who created the concept and developed the products currently under clinical testing by Hemarina.

Early in his career, Franck Zal had the opportunity to collaborate with a renowned scientist, Professor Toulmond, then the director of a laboratory first in Paris at the Sorbonne, and in 1993 moving it to a Marine Biological Station in Roscoff (Brittany, France), which had originally been founded in 1872 by Professor de Lacaze-Duthiers. Between 1993 and 1996, Franck Zal undertook his PhD studies with the mentorship of Prof. Toulmond and began work on *Arenicola marina*. The Lugworm is a very old species since it appeared on earth 450 million years ago (Lucy, the common ancestor of all humanity by contrast, and discovered in Ethiopia, is merely 3.18 million years old); it colonizes today the intertidal area of the marine east-Atlantic shoreline of France from the North-Sea to Biarritz.

Hemarina's research started with a very basic question: how the lugworm can breathe in aqueous and land environments, at high tide and low tide.

Franck Zal studied the blood of this animal and in particular, its hemoglobin. Indeed, hemoglobin is a very old protein, an oxygen carrier that is found even in bacteria, and serves as a bridge between the external environment of the species and its physiological needs. Hence, hemoglobin is a protein of choice to understand physiological adaptations.

Franck Zal categorized the structure of *Arenicola marina* hemoglobin, now called M101 [1–5]. He demonstrated that M101 addresses all of the specifications for a "universal oxygen carrier" that physicians have been seeking for decades. After his PhD, Franck Zal studied for three post-doctorate years (University of California, Santa Barbara and University of Antwerp, Belgium). In 1999, he commenced his academic career at the French National Centre of Scientific Research (CNRS) where he received a 2001 bronze medal for his innovative research in this field. However, since this academic research center was mostly focused on basic science, in 2007, he opted to transform and to create the biotech company HEMARINA with the ambition to develop therapeutic oxygen carriers for saving lives.

Early on, Zal's work was focused on the structure-function relationships of hemoglobin M101. He demonstrated that M101 has unusual properties: (i) it is a naturally extracellular and polymerized hemoglobin; (ii) its molecular weight is

F. Zal (✉) · E. Delpy
HEMARINA SA – Aéropôle centre, Morlaix, France
e-mail: franck.zal@hemarina.com

J. S. Jahr
Department of Anesthesiology and Perioperative Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_34

50-fold that of human Hb; (iii) it has functional O_2-binding and -liberating properties similar to those of human Hb (HbA inside the red blood cell); (iv) it can bind 156 molecules of oxygen, compared to 4 in the case of human HbA; (v) it has naturally antioxidative properties due to an intrinsic superoxide-dismutase-like activity.

Historically, and before the discovery of this natural hemoglobin from *Arenicola marina*, two approaches have been investigated to develop universal oxygen carriers: (i) a chemical method using perfluorocarbons; and (ii) a biological method using human or bovine Hb. In March 2007, Franck Zal created the French biotechnology company named HEMARINA, in order to develop and promote the lugworm hemoglobin M101 as a therapeutic solution, which was seen to possess all of the specifications necessary to become the leading third-generation blood substitute. In addition to the properties outlined above, the main characteristics of M101 are: (i) functional properties are totally independent of secondary molecules, such as 2,3-DPG; (ii) it functions without any chemical modification and no additional treatment; (iii) it has a functional capability at a large range of temperatures (between 4 °C and >30 °C), which is a major and essential advantage over other hemoglobins; (iv) the absence of any vasoconstrictor effects [6], in contrast to all other products developed to date and referred as hemoglobin-based oxygen carriers (HBOCs). The main functional characteristics of M101 are described in (Table 34.1).

As discussed at the beginning of the chapter, *Arenicola marina* is under constant flux by the action of tides. The worm inhabiting the sediment of the intertidal zone is exposed to pronounced fluctuations of environmental conditions that are considered as extreme and even abiotic/lethal. The tidal ebb and flow of the sea cause periodic changes in temperature, salinity and oxygen supply. *Arenicola marina* lives about 10–30 cm deep in the sediment of intertidal flats. During high tide its J-shaped burrow is irrigated by peristaltic movements of its body wall thus providing the animal with oxygen. At low tide when the burrow is immersed, ventilation becomes impossible and the lugworm is exposed to increasing hypoxia. Therefore, *Arenicola marina* is a model of physiological adaptation to intertidal variations of oxygen (regular changes between normoxic conditions at high tide to hypoxic conditions at low tide); the functioning of its hemoglobin is the basis of this adaptation, allowing the worm to get sufficient oxygenation during aqueous high tide, and consequently oxygen deprivation at low tide. The functional oxygen-binding and delivering properties of *Arenicola marina* hemoglobin (M101) is the basis of $HEMO_2$life® medical device for transplantation.

Table 34.1 Functional properties of M101 under human physiological conditions

P50 (O_2 affinity, mmHg)	7.0
n50 (cooperativity)	2.5
Bohr coefficient	−0.5
ΔH (KJ/mol)	−19
COP (mmHg)	1.0
Viscosity (cP)	1.2
SOD activity (U/mg Hb)	3.5
CN inhibition	100%
Fe (atom/molecule)	156
Cu (atom/molecule)	3.6
Zn (atom/molecule)	5.1

Adapted from [5]

Preclinical Investigations

Indeed, ischemia of a graft during organ harvest and preservation followed by reperfusion during transplant may be schematically compared to *Arenicola marina* intertidal oxygen variations. The concept of combination of M101 to existing organ preservation solutions to allow graft oxygenation before transplant naturally emerged and has led to $HEMO_2$life® bioinspired application (Fig. 34.1).

During organ preservation, organs are occluded from the vascular system and therefore interrupt supply in oxygen and nutrients. Hence, cells are rapidly in an anoxic environment, with severe consequences on the cell's physiology. In fact, the low-tide/high tide cycle endured by *Arenicola marina* is similar to the pathophysiological phenomenon called ischemia/reperfusion, more precisely occurring during graft disconnection from the donor and following the graft reperfusion with the recipient.

The concept of supplementing classical organ preservation protocols has rapidly gained interest. Simple addition of a molecule to established protocols represents significant value for translation to the clinical setting, compared to more cumbersome approaches of changing perfusate solutions and/or machines. Current organ preservation techniques revolve around the use of hypothermia, particularly in the context of static preservation, both because of its historical roots and of its ease of implementation. While adequate for grafts harvested 20 years ago, the complication rates are critically increased for extended criteria donors (ECD) and donation after circulatory death donors (DCD) because of the high sensitivity of these organs to ischemia and reperfusion injuries. While the most effective strategy for these organs is to reduce cold ischemia time, the necessary logistics may not be possible. This situation calls for investigation on novel pre-conditioning methods to improve graft quality.

Benefits of M101 supplementation to a range of preservation solutions used in the clinic was first tested by Thuillier et al. [7] and Mallet et al. [8], both *in vitro* in cold stored cultured kidney epithelial and endothelial cells, and *in vivo* using a pig model of kidney auto-transplantation; this spe-

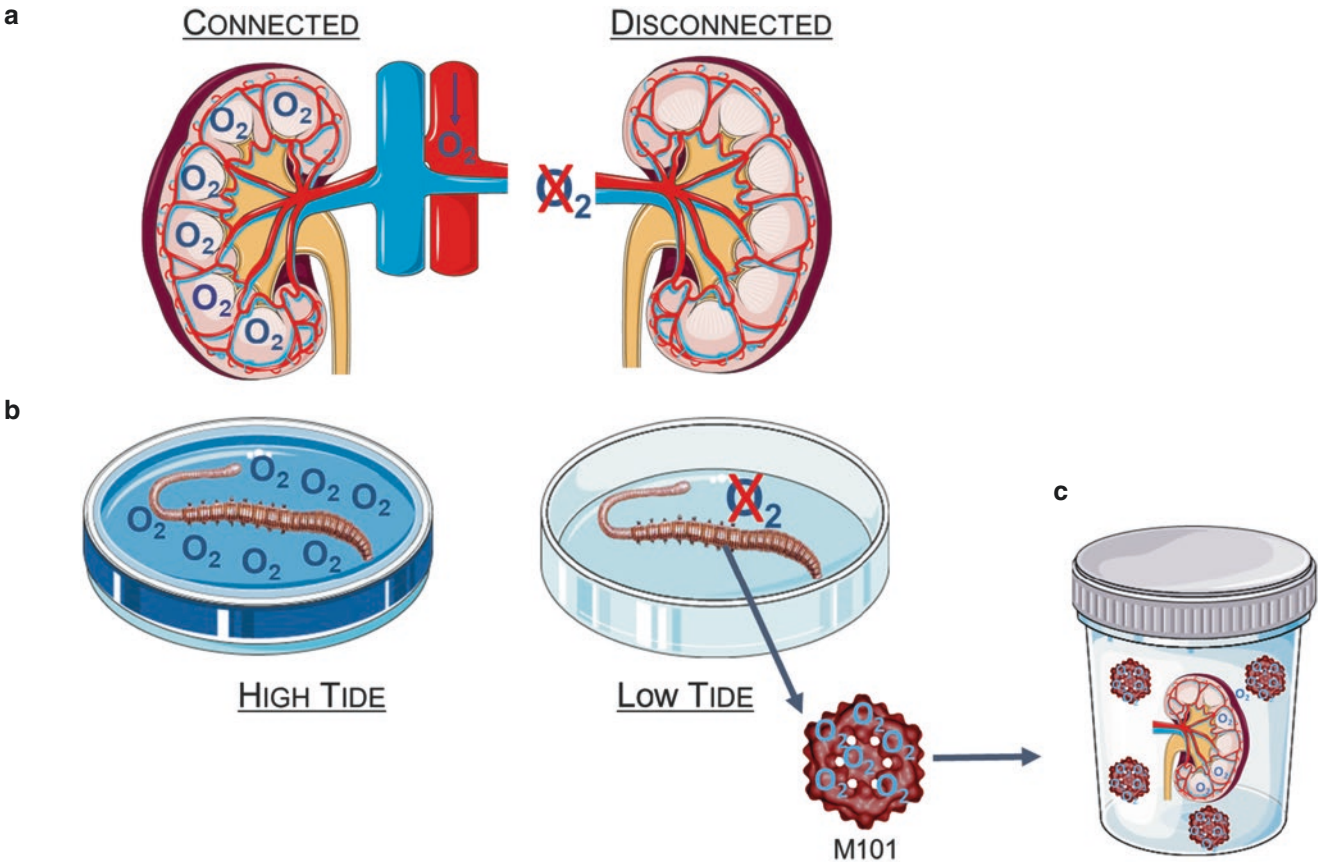


Fig. 34.1 During organ preservation, organs are occluded from the vascular system and therefore interrupt supply in oxygen (**a**). Hence, cells are rapidly in an anoxic environment. During high tide, *Arenicola marina* burrow is irrigated by peristaltic movements of its body wall thus providing the animal with oxygen dissolved in sea water (**b**). At low tide when the burrow is immersed, ventilation becomes impossible and the lugworm is exposed to increasing hypoxia. The functioning of its hemoglobin M101 allows the worm to get sufficient oxygenation during oxygen deprivation at low tide. The comparison of the situation of an organ disconnected from the circulation (organ graft from a donor) and the situation of the marine worm at low tide, together with the comprehension of the functional oxygen-binding and delivering properties of M101, are the basis of HEMO$_2$life® medical device use for organ preservation during transplantation (**c**)

cies presents an elaborate system of interlobular and segmental arteries to supply the numerous kidney lobes, a characteristic shared with humans and higher mammals but absent in rodents or dogs, making porcine models relevant to study human conditions.

Cold static storage of cultured LLC-PK1 cells and human primary aortic endothelium cells during 24 h in different preservation solutions is very deleterious inducing loss of cell viability (LDH release), reduction of metabolic activity and ATP content. Under these experimental conditions mimicking organ preservation, the supplementation by M101 is protective against these events in a dose-dependent manner. In the pig kidney auto transplantation model using CS preservation, early follow-up demonstrated superiority of M101-supplemented solutions, lowering the peak of serum creatinine and increasing the speed of function recovery. On the longer term, supplementation with M101 reduced kidney inflammation level and maintained tissue structural integrity. At the end of the 3-month follow-up period, M101 supplementation proved beneficial in terms of survival and function, as well as slowing the progression of interstitial fibrosis and tubular atrophy, a common cause of chronic loss of graft function and ultimately loss of the graft itself.

Thus, the simple addition of M101 to CS presents an excellent potential for clinical translation. Besides static CS, machine preservation (MP) of grafts has shown mounting interest as it exhibits improvement in graft quality and function. Its ability to get rid of metabolites and cellular waste produced during ischemia has been proposed as the reason for its beneficial effects. However, the presence of these products at reperfusion is most likely associated with the intense activation of the innate immune pathway. In this context, M101 supplementation to MP for O_2 delivery to the most remote graft regions may amplify the organ protection because of the synergistic beneficial effects of M101 and MP [9, 10]. These first accomplishments in kidney transplantation fostered efforts towards other organs [11].

M101 has been studied as a supplementation to the most common lung preservation solution (Perfadex®) in a single lung allotransplantation model in pig [12]. In this study, the addition of M101 during the 24 h hypothermic preservation induced an improvement of functional parameters after transplant: reduction of graft vascular resistance and increase in graft oxygenation. From this study, it can therefore be assumed that the addition of M101 during the preservation period decreases the functional consequences of the second phase of reperfusion (between 2 and 4 h) and improves graft function. Further developments led to a study evaluating the efficacy of M101 in a swine model during an extended lung CS (36 h) followed by 12 h of normothermic ex-vivo lung preservation (EVLP) before lung transplant into a recipient pig [13]. During EVLP assessment, M101-treated lungs showed improvements in physiologic parameters, whereas the control lungs deteriorated. After a total of 48 h of preservation, transplanted grafts in the treatment group displayed significantly better oxygenation (PaO_2/FiO_2) than in the controls. In addition, the use of M101 led to significantly less edema formation, less apoptotic cell death, improved tight junction preservation and lower levels of circulating IL-6 within recipient plasma. Animals show excellent post-transplant outcome, allowing for the possibility for increased preservation times in lung preservation, that may lead to better patient management during the transplant process and a broadened donor pool due to the ability to overcome geographic challenges.

Prior to a heart transplantation procedure, static storage of donor hearts is currently limited to 4–5 h, despite profound hypothermia. Because heart transplantation is an emergency procedure, improved protection to extend safe storage duration would be advantageous. M101 has been used in isolated Langendorff-perfused rat hearts preserved during 8 h in Celsior® solution under static conditions [14] and was shown to improve significantly post-ischemic recovery of heart function (left ventricular developed pressure and coronary blood flow).

Hypothermic oxygenated machine perfusion (HOPE) is a promising technique for providing oxygen to the liver during graft preservation and has been shown to be more effective than CS. However, because of associated logistical constraints and costs, addition of an oxygen transporter to static cold-storage solutions might be easier. The performance of a CS liver graft UW solution supplemented with M101 was tested in an orthotopic pig liver transplantation model [15]. Results demonstrated a beneficial protective effect of M101, with blood levels of ASAT, ALAT and LDH significantly reduced on day 1 post-transplant. M101 effectively oxygenates liver grafts during preservation, preventing post-transplant injury and may therefore be a simplest alternative to the use of machine perfusion.

Pancreas or islet transplantation are life-saving therapies for several diseases (brittle type 1 diabetes, pancreatitis, etc.). However, prolonged pancreas cold ischemia (CI) time is associated with inferior graft survival rates and with poor islet isolation yielding. In this context, delay of CI injury could allow the use of a larger number of pancreases for transplantation, including marginal organs. An experimental study tested the efficacy of M101 in improving pancreas and islets quality [16]. M101 was added to the preservation solution of rat pancreas during ischemia and a resultant decrease in oxidative stress (ROS), necrosis (HMGB1), and cellular stress pathway (p38 MAPK) activity was observed. Freshly isolated islets had improved function when M101 was injected into the pancreas. Additionally, human pancreases exposed to M101 for 3 h showed an increase in complex 1 mitochondrial activity, as well as activation of AKT activity, a cell survival marker. Insulin secretion was also up-regulated in isolated islets. These results demonstrate a positive effect of the oxygen carrier M101 on rat and human pancreas during preservation, with an overall improvement in post-isolation islet quality.

Clinical Applications and Investigations

Preclinical studies have therefore demonstrated the safety of M101 as an additive to organ preservation solutions and its beneficial effect on ischemia/reperfusion injuries. The next step towards the commercialization of the hemoglobin from *Arenicola marina* as a medical device was obviously to evaluate it in humans. With this intention, the product $HEMO_2$life® has been manufactured according to European Union Good Manufacturing Practice (GMP) governing this category of product. The manufacturing process begins by freezing the worm to create a hemorrhagic shock and to release its extracellular hemoglobin (M101). After successive steps of solid/liquid extraction, purification, filtration and gamma irradiation, the final product is a GMP class III medical device containing M101 (Fig. 34.2).

The first clinical study evaluating $HEMO_2$life® added at 1 g/L to the preservation solution of one of two kidneys from the same donor was the multicenter open-label study OXYOP (Evaluation of a Marine OXYgen Carrier: $HEMO_2$life® for hypOthermic Kidney Graft Preservation, Before Transplantation; Clinical Trial Registry No. NCT02652520). All adverse events were analyzed by an independent data and safety monitoring board. Among the 58 donors, 38% were extended criteria donors. Grafts were preserved in cold storage (64%) or machine perfusion (36%). No allergic or hypersensitivity reactions, no infections and no prothrombotic effects related to the product were reported. Preimplantation

Fig. 34.2 M101 is an extracellular hexagonal bilayer hemoglobin (**a**) fully described by Zal et al. in 1997 and extracted from the sea worm *Arenicola marina*. In the natural environment, *Arenicola marina* colonizes the intertidal area on the west coast of France (**b**). For industrial purposes, worms are bred in aquaculture under strict conditions of traceability and reproducibility in a dedicated farm (**c**). $HEMO_2life$® containing the oxygen carrier M101 is a class III medical device under clinical investigation and manufactured according to GMP (**d**). $HEMO_2life$® is meant to be used *ex vivo* as an additive to preservation solutions (**e**). (Adapted from Le Meur et al., 2019 – photos B&C credits Mathieu le Gall)

and 3-month biopsies did not show thrombosis or altered microcirculation. Secondary efficacy end points showed less delayed graft function and better renal function in the $HEMO_2life$® group than in the contralateral kidneys. In the subgroup of grafts preserved in cold storage, Kaplan-Meier survival and Cox regression analysis showed beneficial effects on DGF independent of cold ischemia time. This study was therefore the first study confirming that M101 is safe and shows promising efficacy data in humans [17]. $HEMO_2life$® is currently being tested to evaluate its performance versus standard of care in renal transplantation in a larger multicenter randomized clinical trial OXYOP2 (Clinical Trial Registry No. NCT04181710).

Additional Applications and Indications

$HEMO_2life$® has also been used to preserve a face transplant during a full-face re-transplantation, a world first. Allograft ischemia and its potential sequelae were a major concern in this case due to the geographical distance separating the donor and the recipient. The patient underwent transplantation in January 2018 with a graft preserved with $HEMO_2life$®. In all previous facial transplantation cases, the allografts showed some late revascularization deficits, but this phenomenon was not observed in this patient's case. The surgery was a success and 30 months later, the patient is healthy and the graft has not been rejected [18].

In addition to $HEMO_2$life® developed for organ preservation, several applications for M101 have already been identified since HEMARINA is developing this hemoglobin as a technological platform for new therapeutical applications. Indeed, oxygen is essential for aerobic metabolism of all living cells and oxygen is needed at the level of cells, tissues, organs and the full organism. Based on this postulate, products are developed at all these integrated levels for different intended uses; HEMOXCell® in order to provide *in vitro* cultured cell with physiological oxygen; HEMHealing®, HEMDental-Care® and HEMDental-Regenerativ® for tissue oxygenation and regeneration (wound healing and dental injuries); HEMOXYCarrier® for systemic oxygenation. As an example, the anti-inflammatory and anti-bacterial properties of M101 have been recently reported [19], confirming its pro-healing effects that facilitate the recovery and regeneration of damaged tissues. This proof-of-concept study establishes M101 as a potential therapeutic agent in the context of periodontal wound healing and regeneration. Related to its oxygenation and antioxidant properties, M101 has also been recently proposed as a new therapeutic tool to help to struggle symptomatically the hypoxemia in COVID-19 Patients [20].

In conclusion, all the foregoing is the illustration that applications of the hemoglobin M101 are numerous and almost limitless, acting potentially at all places where oxygen is needed or missing.

Moving Forward

Hemarina's products are revolutionary, in that they are a naturally based hemoglobin and not modified, as are all other hemoglobin-based oxygen carriers and hemoglobin oxygen therapeutic products [21]. This fact makes the products derived from the lugworm hemoglobin, such as $HEMO_2$life®, a game changer, in that it can be formulated to help oxygenate and heal wounds, either from trauma or surgically induced, such as the redo double face transplant [18]. The fact that it has been evaluated in humans for renal transplantation as a preservative for organ preservation also places this hemoglobin in a special category that few others have attained, namely human trials [17].

These revolutionary products carry forty times more oxygen than do red blood cells, it can load and release in any situation where there is tissue hypoxia, and due to the size of each molecule of lugworm hemoglobin, it is 250 times smaller than red blood cells, allowing for transport to areas of poor perfusion or even no perfusion, as long as there is ability for plasma to permeate tissues downstream, that has one of these products in it. It is non antigenic and therefore is a universal donor.

In summary, the hemoglobin derived from lugworms, due to their unique ability to survive both in salt water and on land, for prolonged periods related to their unique hemoglobin, may deliver critical oxygenation to deprived tissues and maintain lifesaving oxygen delivery. Additional indications, such as ARDS from COVID are also being studied and might provide the ability to oxygenate and maintain life, while lung healing may occur without use of ECMO [20].

Key Points

- M101 is a natural extracellular hemoglobin isolated from *Arenicola marina*, a marine worm with 450 million years of evolution.
- M101 extraordinary properties make it a new therapeutic hope for effective oxygenation in the clinic.
- M101 addresses all the specifications of a Universal Oxygen Carrier.
- M101 is the basis of $HEMO_2$life®, a bioinspired medical device for transplantation, with demonstrated beneficial effects on ischemia/reperfusion injuries.
- M101 is a technological platform for providing physiological oxygen: its applications are numerous and almost unlimited.

References

1. Zal F, Green BN, Lallier FH, Vinogradov SN, Toulmond A. Quaternary structure of the extracellular haemoglobin of the lugworm Arenicola marina: a multi-angle-laser-light-scattering and electrospray-ionisation-mass-spectrometry analysis. Eur J Biochem. 1997;243(1-2):85–92.
2. Chabasse C, Bailly X, Rousselot M, Zal F. The multigenic family of the extracellular hemoglobin from the annelid polychaete Arenicola marina. Comp Biochem Physiol B Biochem Mol Biol. 2006;144(3):319–25.
3. Chabasse C, Bailly X, Sanchez S, Rousselot M, Zal F. Gene structure and molecular phylogeny of the linker chains from the giant annelid hexagonal bilayer hemoglobins. J Mol Evol. 2006;63(3):365–74.
4. Rousselot M, Le Guen D, Chabasse C, Zal F. Novel dissociation mechanism of a polychaetous annelid extracellular haemoglobin. FEBS J. 2006;273(7):1582–96.
5. Rousselot M, Delpy E, Drieu La Rochelle C, Lagente V, Pirow R, Rees J-F, et al. Arenicola marina extracellular hemoglobin: a new promising blood substitute. Biotechnol J. 2006;1:333–45.
6. Tsai AG, Intaglietta M, Sakai H, Delpy E, La Rochelle CD, Rousselot M, et al. Microcirculation and NO-CO studies of a natural extracellular hemoglobin developed for an oxygen therapeutic carrier. Curr Drug Discov Technol. 2012;9(3):166–72.
7. Thuillier R, Dutheil D, Trieu MTN, Mallet V, Allain G, Rousselot M, et al. Supplementation with a new therapeutic oxygen carrier reduces chronic fibrosis and organ dysfunction in kidney static preservation. Am J Transplant. 2011;11(9):1845–60.
8. Mallet V, Dutheil D, Polard V, Rousselot M, Leize E, Hauet T, et al. Dose-ranging study of the performance of the natural oxygen transporter HEMO2 Life in organ preservation. Artif Organs. 2014;38(8):691–701.
9. Kasil A, Giraud S, Couturier P, Amiri A, Danion J, Donatini G, et al. Individual and combined impact of oxygen and oxygen trans-

porter supplementation during kidney machine preservation in a porcine preclinical kidney transplantation model. Int J Mol Sci. 2019;20(8):1992.
10. Kaminski J, Hannaert P, Kasil A, Thuillier R, Leize E, Delpy E, et al. Efficacy of the natural oxygen transporter HEMO2life® in cold preservation in a preclinical porcine model of donation after cardiac death. Transpl Int. 2019;32(9):985–96.
11. Thuillier R, Delpy E, Matillon X, Kaminski J, Kasil A, Soussi D, et al. Preventing acute kidney injury during transplantation: the application of novel oxygen carriers. Expert Opin Investig Drugs. 2019;28(7):643–57.
12. Glorion M, Polard V, Favereau F, Hauet T, Zal F, Fadel E, et al. Prevention of ischemia-reperfusion lung injury during static cold preservation by supplementation of standard preservation solution with HEMO2life® in pig lung transplantation model. Artif Cells Nanomed Biotechnol. 2018;46(8):1773–80.
13. Ali A, Watanabe Y, Galasso M, Watanabe T, Chen M, Fan E, et al. An extracellular oxygen carrier during prolonged pulmonary preservation improves post-transplant lung function. J Heart Lung Transplant. 2020;39(6):595–603.
14. Teh ES, Zal F, Polard V, Menasché P, Chambers DJ. HEMO2life as a protective additive to Celsior solution for static storage of donor hearts prior to transplantation. Artif Cells Nanomed Biotechnol. 2017;45(4):717–22.
15. Alix P, Val-Laillet D, Turlin B, Ben Mosbah I, Burel A, Bobillier E, et al. Adding the oxygen carrier M101 to a cold-storage solution could be an alternative to HOPE for liver graft preservation. JHEP Rep. 2020;2(4):100119.
16. Lemaire F, Sigrist S, Delpy E, Cherfan J, Peronet C, Zal F, et al. Beneficial effects of the novel marine oxygen carrier M101 during cold preservation of rat and human pancreas. J Cell Mol Med. 2019;23(12):8025–34.
17. Le Meur Y, Badet L, Essig M, Thierry A, Büchler M, Drouin S, et al. First-in-human use of a marine oxygen carrier (M101) for organ preservation: a safety and proof-of-principle study. Am J Transplant. 2020;20(6):1729–38.
18. Lantieri L, Cholley B, Lemogne C, Guillemain R, Ortonne N, Grimbert P, et al. First human facial retransplantation: 30-month follow-up. Lancet. 2020;396(10264):1758–65.
19. Batool F, Stutz C, Petit C, Benkirane-Jessel N, Delpy E, Zal F, et al. A therapeutic oxygen carrier isolated from Arenicola marina decreased P. gingivalis induced inflammation and tissue destruction. Sci Rep. 2020;10(1):14745.
20. Lupon E, Lellouch AG, Zal F, Cetrulo CL, Lantieri LA. Combating hypoxemia in COVID-19 patients with a natural oxygen carrier, $HEMO_2$life® (M101). Med Hypotheses. 2021;146:110421.
21. HEMARINA institutional video: https://vimeo.com/510177221.

HBOC-201: History, Clinical Trials, and Path Forward

35

Jonathan H. Waters, Jennifer C. Lim,
Joanne M. Blanckenberg, and Jonathan S. Jahr

History

The pursuit of a blood substitute started in 1878 when T. Gaillard Thomas proposed the use of cow's milk as a blood substitute [1]. The idea that it could be used as a blood substitute was related to the thought that milk looked like lymphatic fluid. The first hemoglobin-based blood substitute was proposed by William Amberson in 1933 where he observed that many invertebrate animals have free hemoglobin circulating in their vascular system. He proceeded to do complete blood volume exchanges with free hemoglobin in cats where he observed that the cats would act normally for about 5–6 h after which point, they would die. He proceeded to trial a hemoglobin-saline solution in 14 humans suffering from a variety of anemias [2]. In one case of postpartum hemorrhage, 2300 mL of the hemoglobin saline solution was injected. The patient's blood pressure improved with a return to consciousness. Unfortunately, this patient died 9 days later from renal failure. What was concluded from Amberson's cases was that the hemoglobin saline solution would raise blood pressure and that it transported oxygen; however, renal impairment was seen in half of the 14 cases. In the 1950's, the United States Navy tested a free hemoglobin solution in 47 anemic patients with 12 patients developing renal impairment [3]. In 1969, Bunn and Jandl made the observation that the proximal tubule of the kidney was responsible for the clearance of the αβ dimer of hemoglobin, which resulted from hemoglobin breakdown in circulation and was protective of renal function [4]. They then looked at bis(N-maleimidomethyl) ether to reduce the dissociation of the dimers and prevent the renal excretion of the hemoglobin [5]. This basically started the efforts at developing cross-linking reagents in order to effectively deliver oxygen and to keep the hemoglobin molecule in circulation. The work on cross-linking evolved to use glutaraldehyde as a non-site-specific cross-linking agent [6]. Figure 35.1 shows the molecular structure of HBOC-201 which uses glutaraldehyde as a cross-linking agent. In association with its development, Biopure Corporation was founded in 1984. Biopure

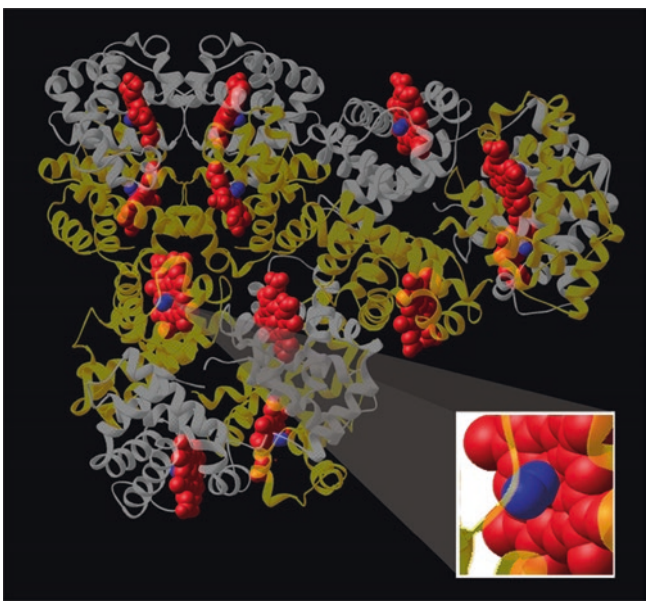


Fig. 35.1 Stroma-free bovine hemoglobin molecules are purified, and glutaraldehyde crosslinked to form a larger protein polymer (green, gray) that has increased vascular retention compared to unmodified stroma-free hemoglobin. The oxygen binding sites are the heme groups (red) that pick-up oxygen (blue) in the lungs and release it to tissues and organs throughout the body

J. H. Waters (✉)
The Departments of Anesthesiology and Bioengineering, University of Pittsburgh, and The McGowan Institute for Regenerative Medicine, Pittsburgh, PA, USA

Department of Anesthesiology, Magee Women's Hospital, Pittsburgh, PA, USA
e-mail: watejh@upmc.edu

J. C. Lim
Boston College, Chestnut Hill, MA, USA

J. M. Blanckenberg
Netcare Milpark Hospital, Parktown, Johannesburg, South Africa

J. S. Jahr
Department of Anesthesiology and Perioperative Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_35

developed two molecules. One was the hemoglobin-glutamer-200 (Oxyglobin®) which received approval in 1997 from the Food & Drug Administration (FDA) for use in dogs with anemia [7]. The second molecule, hemoglobin-glutamer-250 (HBOC-201, HBOC-201®) received approval from the South Africa Medicines Control Council in 2001 and later in Russia yet has yet to gain approval for use in the United States and Europe.

HBOC-201 is a purified cell-free glutaraldehyde cross-linked and polymerized bovine hemoglobin in a modified Ringer's lactate solution (30–35 g hemoglobin/250 mL unit, pH 7.6–7.9, P50 = 40 mmHg). The advantages of HBOC-201 are its ability to be stored at room temperature for up to 3 years; that it does not require cross-matching; and has a circulatory half-life of 19–24 h. Figure 35.2 shows a picture of HBOC-201. Unlike human erythrocytes, it also has an oxygen release independent of 2-3 DPG because oxygen affinity of bovine hemoglobin is regulated by a chloride ion instead of chemical modification by pyridoxylation which is needed by human hemoglobin to decrease its oxygen affinity. Therefore, at physiologic plasma chloride concentrations, polymerized bovine hemoglobin has a physiologic oxygen half-saturation pressure of 40 mmHg that does not diminish with storage. Bovine hemoglobin is purified and then polymerized to decrease its osmotic pressure and increase vascular persistence time, resulting in an HBOC-201 that can carry and unload oxygen in plasma.

Fig. 35.2 Bag of HBOC-201 demonstrating the un-refrigerated form of its storage

However, it is cleared from circulation by the reticuloendothelial system as would any free hemoglobin, and between 5% and 10% is oxidized to form methemoglobin in the plasma, cleared by reductases in the red cell coat, and by other reducing agents. When severe anemia exists, there may not be adequate red cell volume to provide this protective mechanism. Clinically severe methemoglobinemia was not reported in the large trials, however, case reports have indicated that occasional clinically significant methemoglobinemia may occur, requiring methylene blue or other antioxidant treatments [8].

The next section of this chapter is a summary of the clinical trials that have been undertaken to prove safety and efficacy.

Clinical Trials

Table 35.1 summarizes the published HBOC-201 in humans. For this section, a discussion of the most important trials will be undertaken. The first study of importance was the largest study with a total of 668 patients with 350 patients receiving HBOC-201 and 338 patients receiving allogeneic erythrocytes [8, 12]. This multicenter double-blind, randomized multinational study was one of the two largest clinical trials

Table 35.1 Published HBOC-201 studies in humans

Patient population	Total number of patients	FDA study phase
Elective percutaneous coronary revascularization [9]	47 (17 pts got 15 g; 12 pts got 30 g), 16 were placebo	I/II
Post-cardiopulmonary bypass [10]	98 (48 to PRBC, 50 to HBOC)	III
Surgical patients expected to be transfused [11]	81 (26 to LRS, 55 to HBOC)	I/II
Post-abdominal aortic reconstruction [8]	72 (24 to PRBC, 48 to HBOC)	III
Orthopedic surgery [12, 13]	668 (338 to PRBC, 350 to HBOC)	III
Elective abdominal surgery [14]	39 (20 controls, 19 to HBOC)	I/II
Elective liver resection surgery [15]	14 (8 to hetastarch, 6 to HBOC)	I/II
Normal subjects [16]	24 (6 to LRS, 18 to HBOC)	I
Exercising normal subjects [17]	6 (all received HBOC)	I
Sickle cell disease not in crisis [18]	19 (7 to saline, 12 to HBOC)	I/II
Prolonged high dose use [19]	10 patients	

conducted with HBOC products. The purpose of the study was to assess whether or not use of HBOC-201 would reduce allogeneic red blood use in orthopedic patients requiring blood transfusion.

Patients were randomized at the first transfusion decision to receive either HBOC-201 or allogeneic erythrocytes. Transfusion thresholds included a total hemoglobin concentration of less than 10.5 g/dL and a patient needed to have at least one of the following clinical signs: heart rate greater than 100 beats per minute; a systolic blood pressure less than 90 mmHg or less than 70% of preoperative screening value; electrocardiogram evidence of myocardial ischemia; metabolic acidosis (Base Deficit −4 or worse); acute blood loss greater than 7 mL/kg within 2 h or less; oliguria with urine output less than 0.5 mL/kg/h for at least 2 h.

Once treatment was initiated with a 2-unit (500 mL) loading dose of HBOC-201, additional treatment was permitted for up to 6 days to a total of 10 units HBOC-201 (130 g hemoglobin) using the same criteria as for enrollment. Need for continuing transfusion beyond 10 units of HBOC-201 was met by crossing-over HBOC-201 randomized patients to allogeneic erythrocytes. The designs of this Phase III study required that limits be placed on the amount of HBOC-201. The clinical trial scenarios simulated circumstances where HBOC-201 was used until erythrocytes became available to replace moderate (less than 3 units of erythrocytes) blood loss for elective surgery. The primary endpoints were transfusion avoidance and blinded assessment of safety non-inferiority.

Among the 350 HBOC-201 patients in this trial, 96.3% avoided erythrocyte transfusion at Day 1, 70.3% at Day 7 and 59.1% at 6 weeks after surgery. A summary of overall medical risk assessment for the intent to treat, determined by blinded review of patient medical records and adverse events by treatment group, showed the overall odds ratio for adverse events was 1.41–1.43 between groups. Ten deaths occurred in patients randomized to HBOC-201 and six in the patients randomized to allogeneic erythrocytes ($p = 0.450$). No deaths in either treatment group were categorized as being associated with either treatment.

Mean baseline hematocrits of the 350 subjects treated with HBOC-201 and the 338 subjects treated with allogeneic erythrocytes were both 28%. In both treatment groups, nadir hemoglobin was the reason most often documented for the first transfusion decision followed by tachycardia (greater than 100 beats per minute). The primary efficacy endpoint, avoiding erythrocyte transfusions throughout the entire 6-week study period, was achieved with 59.4% of the subjects in the HBOC-201 group. Considering the intent to replace up to 6 units of erythrocytes by up to 10 units of HBOC-201, the actual rate of full blood avoidance was even higher since only 317 (93.8%) of subjects in the erythrocyte arm received 6 or less units.

Examining the laboratory data in the study, the investigators found no difference in acid-base parameters, albumin, total bilirubin, alkaline phosphatase, lactate dehydrogenase, glutamyl transferase, and glucose between treatment groups. However, they did find an elevated total protein at follow-up in the HBOC-201 group, as well as an increase in aspartate aminotransferase and alanine aminotransferase. Lipase was increased in the HBOC-201 group in 5–11% of patients, versus 1–2% of patients in the allogeneic erythrocyte group. Creatinine was increased greater than 25% over baseline in 12 patients (6%) in the HBOC-201 group versus 3 patients (2%) in the allogeneic erythrocytes group.

In patients with moderate transfusion needs, HBOC-201 was shown to significantly reduce allogeneic erythrocyte use without an increase in adverse effects. However, in patients with higher transfusion needs, HBOC-201 failed to correct the anemic condition due in part to an unbalanced study design and inherent limitations of HBOC-201. Considering the more diluted hemoglobin concentration (13 g/dL hemoglobin in 250 mL), HBOC-201 would inherently require more volume than allogeneic erythrocytes. Therefore, in high demand patients for a comparable effect, larger volumes of HBOC-201 would be required which in turn might have led to volume overload and a higher incidence of adverse effects.

In another multicenter, randomized, double-blind study, HBOC-201 and/or allogeneic erythrocytes were administered post cardiac surgery to patients with hemoglobin values between 6.5 and 9.0 g/dL [9]. Patients in the study were first provided with conventional techniques to avoid allogenic erythrocyte transfusion, such as preoperative autologous donations, cell salvage, and antifibrinolytic agents. After meeting transfusion criteria, they were then given up to three blinded doses of either HBOC-201 (first dose 60 g/500 mL, second & third doses 30 g in 250 mL) or allogeneic erythrocytes within 72 h after the decision to transfuse was made. After the 72 h or three transfusions (whichever occurred first), patients in both treatment groups were unblinded and received RBCs if transfusion was still necessary. About a third of patients in the HBOC-201 group did not require any allogenic erythrocyte transfusion. Patients required an average of 2.19 units of allogeneic erythrocytes in the control group compared to 1.72 units in the HBOC-201 group. Thus, there was a mean reduction of 0.47 units of allogeneic erythrocyte units per patient in those who initially received HBOC-201. Also, of note, there was no significant difference between the groups in the numbers of patients requiring postoperative clotting factors. Patients in the HBOC-201 group also had significantly decreased hematocrit and hemoglobin levels on postoperative days (POD) 1, 2, and 3 compared to the control group. However, by POD 6, these variables were equivalent between the two groups. There were also statistically significant decreases in mean

cardiac index and pulse oximetry oxygen saturation and increases in systemic and pulmonary arterial pressure in the HBOC-201 group compared to the control group.

This study suggested that HBOC-201 maintained oxygen transport and that oxygen content was like that for those transfused with allogeneic erythrocytes. HBOC-201 is also known to increase systemic blood pressure through vascular nitric oxide scavenging or from the release of endothelin 1. This may be advantageous specifically for postoperative cardiac surgery patients because they may have low systemic vascular resistance resulting from sedation, use of preoperative vasoactive drugs, or postoperative anemia resulting in decreased blood viscosity, and usually require the use of alpha-agonists to maintain perfusion. Interestingly, hematocrit values were similar between both groups by POD 6, which has been suggested to be due to an effect of HBOC-201 on erythrocyte production by increasing serum iron, ferritin, and erythropoietin levels. This study also highlighted the limitations of HBOC-201, which are its short plasma half-life of less than 24 h and its oxidization to methemoglobin. The study found that 15% of circulating HBOC-201 converted to methemoglobin by POD 1 and 40% by POD 2. Overall, the study concluded that using as much as 120 g of hemoglobin from HBOC-201 resulted in a modest degree of about half a unit of allogenic blood conservation in 2/3rds of the group and avoided allogenic transfusion in 1/3rd of the group.

Another study from a multicenter, randomized, single-blind, parallel-group study looked at the administration of HBOC-201 in noncardiac surgery patients [10]. The study was designed to see how many patients would not need allogenic erythrocyte transfusion after initiation of HBOC-201, which was up to 7 units in the first 6 days. Those requiring subsequent transfusions were given allogeneic erythrocytes. Those in the HBOC-201 group were infused with 2 units (60 g Hb) to match the hemoglobin content in 1 unit of allogeneic erythrocytes. To meet transfusion criteria, patients had to have a total hemoglobin level of at least 2 g/dL less than the estimated discharge hemoglobin or less than 6 g/dL. Transfusions were not permitted if the hemoglobin was greater than 10 g/dL. A transfusion was also allowed if one or more of the following symptoms were present: heart rate greater than 100 bpm, systolic blood pressure less than 90 mmHg, metabolic acidosis (base excess of −4 or worse), acute blood loss greater than 7 mL/kg within a 2-h period, oliguria with urine output of less than 0.5 mL/kg/h for 2+ h, and restricted activity due to weakness or dizziness. On average, the HBOC-201 group received 3.2 units compared to 4.4 units in the control group. The HBOC-201 group showed a transient increase in blood pressure which lasted up to 2 days following administration. The greatest difference between the groups was the incidence of jaundice though these incidences were not associated with liver failure. The prehepatic jaundice correlated with an increased bilirubin load that was consistent with the processing of HBOC-201 to bilirubin. There was no difference in significant adverse effects between the two groups. Results also showed a decrease in hematocrit with HBOC-201 transfusion compared to an increase with allogeneic erythrocytes due to hemodilution in the erythrocyte-absent HBOC-201 group. There was also an increase in total hemoglobin for both groups, but more so with allogeneic erythrocyte transfusion suggesting a significantly lower Hb concentration of HBOC-201 compared to allogeneic erythrocytes.

The authors concluded that administering up to 7 units of HBOC-201 over the span of 6 days averted allogeneic transfusion in about 43% of patients who would have otherwise necessitated erythrocyte transfusion. However, results here may have been subject to design bias, such as undertreating the HBOC-201 that would lead to an overestimation of HBOC-201 efficacy. On the other hand, HBOC-201 associated adverse events may have been over-reported due to assessment differences. Another important flaw in the study was that transfusion was recommended for hemoglobin values between 6 and 10 g/dL. With such a wide and ambiguous range, it makes it difficult to accurately assess the impact of HBOC-201 on reduction and avoidance of allogeneic erythrocyte transfusion. This, however, is not a fault of the trial design, but due to poorly defined and practiced transfusion protocols that have existed over the past few decades. Patients in the HBOC-201 group were maintained with a total hemoglobin concentration average of about 1 g/dL lower and a hematocrit 6% lower than the allogeneic transfusion group. Therefore, it is unclear if the HBOC-201 group was undertreated or patients in the allogeneic group were overtreated.

Most recently, a retrospective observational cohort study of critically ill patients was performed on patients who received a total of 10 or more units of HBOC-201. This was performed as an investigational medication under the FDA expanded access program from May 2014 through December 2017. This study was unique in that it was conducted outside of a clinical trial and involved larger doses of HBOC-201 for prolonged periods. Of the 41 patients who were treated with HBOC-201 during this time, only 10 patients received 10 or more units. Ten units was the cut-off minimum because 10 units was the maximum number of units given in previous trials. All except one patient declined blood due to religious beliefs. The one patient who did not receive allogeneic erythrocyte transfusion was because no compatible blood was available. The mean native hemoglobin concentration was 3.3 g/dL (standard deviation = 0.9 g/dL) with transfusion of HBOC-201 occurring within 24 h of signing consent. Patients received an average of 16.2 units (standard deviation = 5.7 g/dL) over the course of their treatment. This averaged about 1.99 units/day (standard deviation = 0.17 g/dL). Treatment lasted about 8.2 days (standard deviation = 3 days)

and ended with a total hemoglobin concentration of 7.3 g/dL (standard deviation = 1.7 g/dL). Again, the most common side effects seen were methemoglobinemia and elevated blood pressure. All patients survived to hospital discharge without any significant long-term effects. This study showed that transfusion with HBOC-201 had a remarkable outcome with no mortality and patients being released from the hospital shortly after transfusion. In comparison to a similar study of anemic patients with nadir Hb concentration of 3.3 g/dL, there was a 50% mortality in those who were not given any transfusion. With the assumption of an average circulatory volume of 5 L, the patients in the study were transfused with HBOC-201 that was 80% of their total blood volume. It is also incredible that even with this much product transfused, these critical care patients showed no significant adverse events and fully recovered without any long-term anemia-related morbidities.

Future Work

In July 2002, Biopure, the developer of HBOC-201, submitted a biologics license application to the Food and Drug Administration (FDA). Later the following year, the company also applied to perform clinical trials in human trauma victims. In the summer of 2003, Biopure had their request for a trauma trial and for approval of their biologics license application rejected. In 2009, the company ceased operations and sold its assets to OPK BioTech (Cambridge, MA) who then, in 2014, sold it to Hemoglobin-Oxygen Therapeutics (Souderton, PA), who continues to market the HBOC-201 in South Africa and has generously made it available to patients in the US, in whom blood is not an option (see section below).

Expanded Access

The FDA has allowed HBOC-201 use under an expanded access and compassionate use, meaning it can be used under a research protocol and supervised by an institutional review board. It can be used in situations in which all other options of transfusion therapy have been exhausted and the patient is experiencing severe and dangerous anemia. Typically, these patients come from the Jehovah's Witness faith or are patients that have been heavily alloimmunized and no compatible blood can be found. This latter group are patients with sickle cell disease who have been repetitively transfused. Many patients with life-threatening anemia have recovered fully, although not all patients survive due to their pre-existent disease or their trauma. For example, Donahue et al. [20] describe a 36-year-old Jehovah's witness patient with acute lymphoblastic leukemia who developed a severe anemia (hemoglobin of 3.1 g/dL). Fifteen units of HBOC-201 were administered during induction chemotherapy from which she survived to be discharged. Another example is a 21-year-old Jehovah's witness who suffered a traumatic brain injury [21]. Six days after his injury his hemoglobin had drifted down to 3.5 g/dL. Four units of HBOC-201 were given. On hospital day 9, the patient had a brain herniation and died. A third example was that of a 23-year-old who was crushed by two cars [22]. His hemoglobin nadired at 3.9 g/dL. Seven units of HBOC-201 were administered over 6 days. He was discharged home on day 12. Many examples such as these can be found in the literature where severe anemia is bridged by HBOC-201 (Table 35.2). These examples are also illustrative of how people can survive with anemias at levels which most medical providers view as not survivable.

Current Use

Given the high HIV infection rate in South Africa, the supply and availability of uninfected, safe blood was not guaranteed. This prompted accelerated approval of HBOC-201 by the South African Medicines Control Council in the hope that it would help alleviate problems with severe obstetric hemorrhage, particularly in the rural, under-resourced areas of the country.

Interested doctors and nurses were trained in the use of HBOC-201 by Hemopure SA. On completion of the training program, physicians could access HBOC-201 from the company for use in appropriate patients. The final decision to administer HBOC-201 to any patient is made by the attending physician.

Adult surgical patients, diagnosed with anemia resulting from either pre-existing medical conditions, or secondary to surgical treatment, received HBOC-201 as part of the treatment of their acute. These patients were deemed to have reached a "transfusion trigger". In the absence of HBOC-201, they would have received a red cell transfusion of a minimum of two units.

After an initial 30 g dose of HBOC-201 is administered over 1–3 h, a second dose is administered, depending on the clinical response, severity of the anemia and the level of plasma hemoglobin achieved after the first dose. If, after administration of the first 30 g dose, clinical stability is noted then, the patient would receive a second 30 g dose on the same day if signs or symptoms of ongoing anemia existed. If the patient's hemoglobin concentration was less than 7.0 g/dL, or was expected to decrease to this level, a second unit of HBOC-201 was administered straight after the first unit.

Plasma hemoglobin was measured 1 h after completion of each unit on the first day, and then daily thereafter whilst treatment continued.

Table 35.2 Summary of compassionate use of Hb-based oxygen carriers in patients for whom blood is not an option

Author, publication year	Patient N =	Population	Nadir Hb (g/dL)	Units of HBOC-201 received (units)	Outcome
Davis et al., 2018 [23]	3	Sickle cell (2 Jehovah's witness patients)	3.6–3.8	6–27	All survived to discharge
Donahue et al., 2010 [20]	1	Jehovah's witness with acute lymphoblastic leukemia	3.1	15	Completed chemotherapy and discharged
Fitzgerald et al., 2011 [24]	1	Jehovah's witness trauma patient	2.9	5	Discharge to rehabilitation facility
Jordon and Alexander, 2013 [25]	1	Jehovah's witness with autoimmune hemolytic anemia	2.8	2	Discharge to home
Lundy et al., 2014	1	Jehovah's witness burn patient	5	6	Death from multiorgan failure
Mackenzie et al., 2010 [26]	54	Jehovah's witness patients with severe anemia	3.9 median	8 median units received	Survival of 41.8%, no adverse events attributed to HBOC
Posluszny and Napolitano, 2016 [22]	1	Jehovah's witness trauma patient	3.9	7	Discharged home
Marinaro et al., 2009 [21]	1	Jehovah's witness trauma patient	3.5	6	Brain herniation and death

Administration of additional units of HBOC-201 is determined by clinical signs namely: pulse rate, urine output and the presence of fatigue (especially if the hemoglobin was less than 8 g/dL). Additional doses are administered not only according to the hematocrit of the patient, but also according to clinical evidence and symptoms of anemia. HBOC-201 treatment is discontinued once the patient has attained clinical stability, either due to his/her own red cell hemoglobin concentrations recovering, or normal compensatory mechanisms operating sufficiently. In the South African usage, a maximum of seven units of HBOC-201 per patient was recommended. In patients who remained clinically unstable after the seventh unit of HBOC-201, administration of red blood cells, where permissible, was recommended.

A report was compiled to document the results of a surveillance and educational program of HBOC-201 administered to anemic patients in South Africa from 2002 until 2005. This was not a clinical trial, but rather a post-marketing study. Having made the decision to administer HBOC-201 to a particular patient, it is incumbent on the treating doctor to record the patient's diagnosis, indication for product usage, surgical procedure (if any) and quantity of product used. Supply of product was conditional on agreement by the treating physician to report to the product distributor any SAE or mortality that may have ensued. SAE's were investigated to establish a possible causal relationship to HBOC-201. Only severe adverse events that were directly product related, were documented.

Future Directions

HBOC-201 has been studied as an ex vivo perfusate to preserve, refurbish and facilitate evaluation of extended criteria donor organs. Fontes et al. [27] published the first study to evaluate HBOC-201 as the oxygen-carrying component in a novel liver perfusion solution. Donated livers are increasingly being recovered and maintained prior to transplantation using normothermic ex situ liver perfusion technology [28, 29]. de Vries and colleagues [30] extended these findings to a liver transplant trial that enabled successful grafts in all recipients. Other organs that have used HBOC-201 for organ preservation include kidneys [31], Heart [32], and limb [33]. Additional research to optimize donor organ perfusion with HBOC-201 should expand the pool of available livers and other organs for transplantation to seriously ill, wait-listed recipients.

Lastly, the United States Department of Defense in conjunction with the University of Stellenbosch in South Africa is conducting a clinical trial to evaluate the use of HBOC-201 in conjunction with freeze-dried plasma in pre-hospital trauma patients [34]. If this study is found to be lifesaving, it could potentially place HBOC-201 in every ambulance.

Summary

Guidelines and best practices for HBOC-201 administration have been established [35]. Multiple lines of evidence dispel the myth of an intrinsic HBOC-201 toxicity. Evidence consists of a beneficial effect include nonclinical studies, clinical trials (phase II, III), compassionate use or expanded access, and donor organ perfusion. HBOC 201 is an effective "oxygen bridge" because it can be used to delay or avoid allogeneic erythrocyte transfusion in acute anemia. It is also a safe alternative to allogeneic erythrocytes when blood is not an option or available. The HBOC-201 body of published evidence that adverse events associated with randomization to HBOC-201 in an early phase III clinical trial were due to under treatment of anemia and patient circulatory volume mismanagement rather than an intrinsic toxicity. Adverse effects of HBOC-201 may be managed by use of published paradigms and standard pharmacotherapy.

Key Points

1. Hemopure is the most tested HBOC to date and room temperature stable for 3 years
2. Hemopure is available for human use in South Africa and Russia for surgical anemia
3. Hemopure is available for Expanded Use when blood is unavailable or not an option
4. Hemopure has been tested for cardiac safety in animal and human models and deemed safe
5. Hemopure has been evaluated as a perfusate in liver transplant preservation and in multiple other indications and appears safe and effective

Conflicts of Interest The author, JHW, is a member of the Advisory Committee for Haemonetics, Inc. and a consultant for LivaNova, Inc.

References

1. Thomas TG. The intravenous injection of milk as a substitute for the transfusion of blood. NY State J Med. 1878;47:449–65.
2. Amberson WR, Jennings JJ, Rhode CM. Clinical experience with hemoglobin-saline solutions. J Appl Physiol. 1949;1:469–89.
3. Winslow RM. The results of 62 large-volume hemoglobin infusions in man. Hemoglobin-based red cell substitutes. Baltimore/London: Johns Hopkins University Press; 1992. p. 177–8.
4. Bunn HF, Jandl JH. The renal handling of hemoglobin: II. Catabolism. J Exp Med. 1969;129:925–34.
5. Bunn HF, Esham WT, Bull RW. The renal handling of hemoglobin. I. Glomerular filtration. J Exp Med. 1969;129:909–23.
6. Harris DR, Palmer AF. Modern cross-linking strategies for synthesizing acellular hemoglobin-based oxygen carriers. Biotechnol Prog. 2008;24(6):1215–25. https://doi.org/10.1002/btpr.85.
7. Jahr JS, Moallempour M, Lim JC. HBOC-201, hemoglobin glutamer-250 (bovine), HBOC-201 (Biopure Corporation). Expert Opin Biol Ther. 2008;8(9):1425–33. https://doi.org/10.1517/14712598.8.9.1425.
8. Van Hemelrijck J, Levien LJ, Veeckman L, Pitman A, Zafirelis Z, Standl T. A safety and efficacy evaluation of hemoglobin-based oxygen carrier HBOC-201 in a randomized, multicenter red blood cell controlled trial in noncardiac surgery patients. Anesth Analg. 2014;119(4):766–76. https://doi.org/10.1213/ANE.0000000000000305.
9. Serruys PW, Vranckx P, Slagboom T, et al. Haemodynamic effects, safety, and tolerability of haemoglobin-based oxygen carrier-201 in patients undergoing PCI for CAD. EuroIntervention. 2008;3(5):600–9. https://doi.org/10.4244/eijv3i5a108.
10. Levy JH, Goodnough LT, Greilich PE, et al. Polymerized bovine hemoglobin solution as a replacement for allogeneic red blood cell transfusion after cardiac surgery: results of a randomized, double-blind trial. J Thorac Cardiovasc Surg. 2002;124(1):35–42. https://doi.org/10.1067/mtc.2002.121505.
11. Sprung J, Kindscher JD, Wahr JA, et al. The use of bovine hemoglobin glutamer-250 (HBOC-201) in surgical patients: results of a multicenter, randomized, single-blinded trial. Anesth Analg. 2002;94(4):799–808, table of contents. https://doi.org/10.1097/00000539-200204000-00006.
12. Jahr JS, Mackenzie C, Pearce LB, Pitman A, Greenburg AG. HBOC-201 as an alternative to blood transfusion: efficacy and safety evaluation in a multicenter phase III trial in elective orthopedic surgery. J Trauma. 2008;64(6):1484–97. https://doi.org/10.1097/TA.0b013e318173a93f.
13. Jahr JS, Liu H, Albert OK, et al. Does HBOC-201 (HBOC-201) affect platelet function in orthopedic surgery: a single-site analysis from a multicenter study. Am J Ther. 2010;17(2):140–7. https://doi.org/10.1097/MJT.0b013e3181a2b08d.
14. Kasper SM, Grune F, Walter M, Amr N, Erasmi H, Buzello W. The effects of increased doses of bovine hemoglobin on hemodynamics and oxygen transport in patients undergoing preoperative hemodilution for elective abdominal aortic surgery. Anesth Analg. 1998;87(2):284–91. https://doi.org/10.1097/00000539-199808000-00009.
15. Standl T, Burmeister MA, Horn EP, Wilhelm S, Knoefel WT, Schulte am Esch J. Bovine haemoglobin-based oxygen carrier for patients undergoing haemodilution before liver resection. Br J Anaesth. 1998;80(2):189–94. https://doi.org/10.1093/bja/80.2.189.
16. Hughes GS Jr, Francome SF, Antal EJ, et al. Hematologic effects of a novel hemoglobin-based oxygen carrier in normal male and female subjects. J Lab Clin Med. 1995;126(5):444–51. Cited in: Ovid MEDLINE(R) at http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=med3&NEWS=N&AN=7595029. Accessed January 28, 2021.
17. Hughes GS Jr, Yancey EP, Albrecht R, et al. Hemoglobin-based oxygen carrier preserves submaximal exercise capacity in humans. Clin Pharmacol Ther. 1995;58(4):434–43. https://doi.org/10.1016/0009-9236(95)90057-8.
18. Gonzalez P, Hackney AC, Jones S, et al. A phase I/II study of polymerized bovine hemoglobin in adult patients with sickle cell disease not in crisis at the time of study. J Investig Med. 1997;45(5):258–64. Cited in: Ovid MEDLINE(R) at http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=med4&NEWS=N&AN=9249998. Accessed January 28, 2021.
19. Zumberg M, Gorlin J, Griffiths EA, et al. A case study of 10 patients administered HBOC-201 in high doses over a prolonged period: outcomes during severe anemia when transfusion is not an option. Transfusion. 2020;60(5):932–9. https://doi.org/10.1111/trf.15778.
20. Donahue LL, Shapira I, Shander A, Kolitz J, Allen S, Greenburg G. Management of acute anemia in a Jehovah's Witness patient with acute lymphoblastic leukemia with polymerized bovine hemoglobin-based oxygen carrier: a case report and review of literature. Transfusion. 2010;50(7):1561–7. https://doi.org/10.1111/j.1537-2995.2010.02603.x. Epub 2010 Apr 23. PMID: 20456679.
21. Marinaro J, Smith J, Tawil I, Billstrand M, Crookston KP. HBOC-201 use in traumatic brain injury: case report and review of literature. Transfusion. 2009;49(10):2054–9. https://doi.org/10.1111/j.1537--2995.2009.02235.x. Epub 2009 May 27. PMID: 19497051.
22. Posluszny JA, Napolitano LM. Hemoglobin-Based Oxygen Carrier for Traumatic Hemorrhagic Shock Treatment in a Jehovah's Witness. Arch Trauma Res. 2016;5(2):e30610. https://doi.org/10.5812/atr.30610. PMID: 27679789; PMCID: PMC5035516.
23. Davis JM, El-Haj N, Shah NN, et al. Use of the blood substitute HBOC-201 in critically ill patients during sickle crisis: a three-case series. Transfusion. 2018;58:132–7.
24. Fitzgerald MC, Chan JY, Ross AW, et al. A synthetic haemoglobin-based oxygen carrier and the reversal of cardiac hypoxia secondary to severe anaemia following trauma. Med J Aust. 2011;194:471–3.
25. Jordan SD, Alexander E. Bovine hemoglobin: a nontraditional approach to the management of acute anemia in a Jehovah's Witness patient with autoimmune hemolytic anemia. J Pharm Pract. 2013;26:257–60.
26. Mackenzie CF, Moon-Massat PF, Shander A, Javidroozi M, Greenburg AG. When blood is not an option: factors affecting survival after the use of a hemoglobin-based oxygen carrier in 54 patients with life-threatening anemia. Anesth Analg. 2010;110:685–93.

27. Fontes P, Lopez R, van der Plaats A, Vodovotz Y, Minervini M, Scott V, Soltys K, Shiva S, Paranjpe S, Sadowsky D, Barclay D, Zamora R, Stolz D, Demetris A, Michalopoulos G, Marsh JW. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. Am J Transplant. 2015;15(2):381–94. https://doi.org/10.1111/ajt.12991. PMID: 25612645; PMCID: PMC5024042.
28. Laing RW, Bhogal RH, Wallace L, Boteon Y, Neil DAH, Smith A, Stephenson BTF, Schlegel A, Hübscher SG, Mirza DF, Afford SC, Mergental H. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. Transplantation. 2017;101(11):2746–56. https://doi.org/10.1097/TP.0000000000001821. PMID: 28520579; PMCID: PMC5656179.
29. Matton APM, Burlage LC, van Rijn R, de Vries Y, Karangwa SA, Nijsten MW, Gouw ASH, Wiersema-Buist J, Adelmeijer J, Westerkamp AC, Lisman T, Porte RJ. Normothermic machine perfusion of donor livers without the need for human blood products. Liver Transpl. 2018;24(4):528–38. https://doi.org/10.1002/lt.25005. Erratum in: Liver Transpl. 2018 Aug;24(8):1151. PMID: 29281862; PMCID: PMC5900573.
30. de Vries Y, Berendsen TA, Fujiyoshi M, et al. Transplantation of high-risk donor livers after resuscitation and viability assessment using a combined protocol of oxygenated hypothermic, rewarming and normothermic machine perfusion: study protocol for a prospective, single-arm study (DHOPE-COR-NMP trial). BMJ Open. 2019;9(8):e028596. Published 2019 Aug 15. https://doi.org/10.1136/bmjopen-2018-028596.
31. Bhattacharjee RN, Patel SVB, Sun Q, Jiang L, Richard-Mohamed M, Ruthirakanthan A, Aquil S, Al-Ogaili R, Juriasingani S, Sener A, Luke PPW. Renal protection against ischemia reperfusion injury: hemoglobin-based oxygen carrier-201 versus blood as an oxygen carrier in ex vivo subnormothermic machine perfusion. Transplantation. 2020;104(3):482–9. https://doi.org/10.1097/TP.0000000000002967. PMID: 31568396.
32. White CW, Hasanally D, Mundt P, Li Y, Xiang B, Klein J, Müller A, Ambrose E, Ravandi A, Arora RC, Lee TW, Hryshko LV, Large S, Tian G, Freed DH. A whole blood-based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. J Heart Lung Transplant. 2015;34(1):113–21. https://doi.org/10.1016/j.healun.2014.09.021. Epub 2014 Sep 28. PMID: 25447577.
33. Said SA, Ordeñana CX, Rezaei M, Figueroa BA, Dasarathy S, Brunengraber H, Rampazzo A, Gharb BB. Ex-vivo normothermic limb perfusion with a hemoglobin-based oxygen carrier perfusate. Mil Med. 2020;185(Suppl 1):110–20. https://doi.org/10.1093/milmed/usz314. Erratum in: Mil Med. 2020 Sep 18;185(9–10):e1900. PMID: 32074378.
34. HBOC-201® to be evaluated in groundbreaking clinical study of trauma patients. PRNewswire. November 8, 2018. Accessed 27 Jan 2021. https://www.hbo2therapeutics.com/post/HBOC-201-to-be-evaluated-in-groundbreaking-clinical-study-of-trauma-patients-in-the-prehospital-set.
35. Mer M, Hodgson E, Wallis L, Jacobson B, Levien L, Snyman J, Sussman MJ, James M, van Gelder A, Allgaier R, Jahr JS. Hemoglobin glutamer-250 (bovine) in South Africa: consensus usage guidelines from clinician experts who have treated patients. Transfusion. 2016;56(10):2631–6. https://doi.org/10.1111/trf.13726. Epub 2016 Sep 23. PMID: 27658499.

Perftoran: History, Clinical Trials, and Pathway Forward

36

Gary W. Latson

Introduction

Perftoran™ is an oxygen carrying intravenous solution comprised of two perfluorocarbons (PFCs) emulsified by a copolymer and mixed in an electrolyte solution with an overall osmotic pressure similar to plasma. It was originally developed in Russia for use as an oxygen-carrying plasma additive for massive blood loss anemia. It has subsequently been studied and used in multiple other conditions in which improvement in oxygen delivery is believed to be beneficial. It has reportedly been used in over 30,000 human patients with moderate side effects and significant benefits (Maslennikov I, Thompson D, July 2017, Scientific productive company Perftoran (Russia) and FluorO2 Therapeutics INC, Wake Forest NC, personal communication). This chapter will review the pharmacology and physiology, history, clinical trials and clinical experience, and potential future applications of the product.

History

Research and development of PFC-based oxygen carriers began in the Soviet Union in the 1970s by multiple research teams and scientists with eventual consolidation of efforts within the All-Union Scientific Industrial Programme in 1980. An original formulation for Perftoran, including the PFC compounds Perfluorodecalin (PFD) and Perfluoro-N-(4-methylcyclohexyl)-piperidine (PFMCP) was suggested by Kiril Makarov and Lev Gervitz at the Institute of Elemental Organic Compounds. After further development and refinement of the emulsification technology, clinical trials were conducted from 1984 to 1994, and the formulation was registered in Russia in 1996 and later in Ukraine and Kazakhstan. The most common usage in Russia was for blood loss anemia, and polytrauma, but it also showed utility in limb ischemia, traumatic brain injury, organ transplantation, cardiac surgery, burns, and as a topical wound treatment [1].

A U.S. Patent was issued in 2003 describing the manufacture and potential uses of the product [2]. Application to the U.S. FDA has not yet been made (Maslennikov I, Thompson D, July 2017, Scientific productive company Perftoran (Russia) and FluorO2 Therapeutics INC, Wake Forest NC, personal communication). A European Patent Application was filed in 2006 [3].

Perftoran was evaluated and approved in Mexico in 2005, and marketed by KEM Laboratories under the brand name Perftec™, primarily to reduce the need for human packed red blood cells. A clinical trial in Mexico City demonstrated effectiveness in reduction of blood utilization in cardiac surgery and showed a safety and side effect profile consistent with that reported in the Russian literature [4]. To this author's knowledge, this was the only human clinical trial reported in the Western Hemisphere. The approval for use in Mexico was suspended in 2010 due to concerns over the lack of documentation of "Good Manufacturing Practice" (GMP) certification in Russia. Manufacture was suspended in Russia in 2011 and plans were developed to manufacture the product in the USA under GMP certification and re-apply for approval in Mexico and other countries. This has not yet occurred due to lack of investment capital.

Composition and Pharmacology

Composition of Perftoran is detailed in Table 36.1. It is comprised of two primary PFC's (PFD and PFMCP) emulsified with the copolymer Proxanol 268 in a buffered aqueous solution. Small amounts of other PFC compounds ("satellites" or "co-products) are also present. Proxanol 268 is an adduct of polyethylene oxide and polypropylene oxide.

G. W. Latson (✉)
Neurosurgical Anesthesiology, Baylor Scott and White Temple Memorial Hospital, Temple, TX, USA

Texas A&M University College of Medicine, Bryan, TX, USA
e-mail: Gary.latson@bswhealth.org

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_36

Table 36.1 Perftoran composition and properties [1, 5, 6]

Component	Per 100 ml solution
Perfluorodecalin (PFD)	7.0 ml (6.5–7.0 V%)
Perfluoromethylcyclohexylpiperidine (PFMCP)	3.0 ml (3.0–3.5 V%)
Proxanol 268	4.0 g
NaCl	0.6 g
KCL	0.039 g
$MgCL_2$	0.019 g
$NaHCO_3$	0.065 g
NaH_2PO_4	0.02 g
Glucose	0.2 g
H_2O	100 ml
F^-	10 *u*M
Osmotic pressure	300 mOsm
pH	7.3
Viscosity	2.3 cPs
Average particle size	0.06 *u*m (60 nm)

Perftoran, along with Fluosol DA, is considered a "first generation" PFC product. It has lower concentration of PFC and, therefore, lower oxygen carrying capacity, than "second generation" products, including Oxygent, Oxycyte, and Oxyfluor.

The main PFC component of Perftoran, PFD, is also the main component in an earlier PFC product, Fluosol DA™, which was developed in Japan by Green Cross Corporation and approved in multiple countries, including the USA in 1989 for use during coronary angioplasty [7]. While there are similarities between Perftoran and Fluosol DA, there are important differences. PFC compounds are unique in that they are both hydrophobic and lipophobic and must therefore be emulsified for use in human plasma. There are complex interactions between different PFC compounds of varying molecular weights and structure which affect vapor pressure, oxygen carrying capacity, emulsion stability, and retention in tissue. Combinations of two or more PFC compounds have advantages in overall stability and function of the products [8]. Fluosol DA and Perftoran both utilize two PFC compounds with PFD as the primary PFC. Fluosol DA uses a different secondary PFC, perfluorotripropylamine (PFTPA), instead of PFMCP. Small amounts of PFC coproducts are also noted to be present [1].

Emulsification agents also differ; Perftoran uses a copolymer, Proxanol P268 (also known as Poloxamer 268) and Fluosol DA used a different copolymer, Pluronic 68 with egg yolk phospholipid (EYP), and hydroxyethyl starch as an oncotic agent. Pluronic 68 is suspected of causing complement activation and may be partly responsible for the side effects of Fluosol DA [7]. Proxanol 268 reportedly has fewer side effects and less toxicity [1]. Second generation products Oxygent and Oxycyte use mainly EYP as the emulsification agent.

Fluosol must be stored frozen in three separate components and when thawed and mixed is only stable for a few hours [7]. Perftoran is stored frozen as a single solution and can be thawed and remains stable for up to 2 weeks under refrigeration [1]. Oxygent and Oxycyte using EYP are stable at room temperature.

Particle size is hypothesized to be a major determinate of some side effects, including complement activation and thrombocytopenia, with smaller particle size being associated with fewer side effects [8]. Perftoran has the smallest particle size of any of the products mentioned. Advances in their emulsification process results in average particle size of 0.06–0.07 *u*m (60–70 nm) compared to 0.12 *u*m average particle size for Fluosol DA and 0.16–0.2 *u*m for Oxygent and Oxycyte. It is reported that side effects are reduced from 8–10% with Fluosol DA to 3–4% with Perftoran [1, 8].

Perftoran is packaged in a single bottle and is stored frozen at −18 °C to −4 °C for up to 3 years. After controlled thawing at room temperature, it is stable for up to 2 weeks in refrigeration at 4 °C [1]. Re-freezing was originally allowed but was later discouraged as it was felt to increase the rate of side effects.

Perftoran is administered by intravenous infusion in doses ranging from 2 to 30 ml/kg body weight. It is recommended that a small "test dose" of a few ml be administered with a 10–15 min observation period to reveal hypersensitivity reactions (see side effects below). For hemorrhagic anemia, the dose of Perftoran is adjusted for the degree of blood loss. For example, with blood loss of <750 ml, the recommended dose of Perftoran is 200–300 ml along with crystalloid solution. For blood loss of >2000 ml (>40% circulating blood volume), 1000–1500 ml of Perftoran is recommended along with crystalloids, colloids, plasma and red blood cells if available. Additional Perftoran can be given as needed. In order to maximize oxygen delivery, it is recommended that patients receive 40–60% inspired oxygen during Perftoran treatment [6].

Perftoran is distributed in the plasma and extracellular fluids with some cellular uptake by macrophages. The two primary PFC components have different distributions and eliminations. Most PFC is primarily excreted via the lungs (90%) and small amounts are eliminated by evaporation through the skin and by the liver in bile. The half-life in circulating blood is approximately 24 h. Approximately 20–30% is temporarily taken up by macrophages in the liver, spleen, bone marrow and lymphoid tissues, with a terminal elimination half life of 14 days for PFD and 90 days for PFMCP [5]. Proxanol P268 is biologically inert and is eliminated via the kidneys within approximately 24 h.

The toxicity of Perftoran is low. In mice, the LD50 by intravenous injection is 140 ml/kg, or in grams, LD50 of

35.3 g/kg. Chronic toxicity studies in rats and rabbits demonstrated small perivascular infiltrates in the lungs, and vacuolized macrophages in the liver spleen and lymph nodes. The permissible dose in humans has been established at 20 ml/kg (although larger doses have been given). While present in the tissues, PFC compounds are biologically and chemically inactive and long-term studies have shown no evidence of organ pathology, or carcinogenic effects (Maslennikov I, Thompson D, July 2017, Scientific productive company Perftoran (Russia) and FluorO2 Therapeutics INC, Wake Forest NC, personal communication). More than 200 patients treated with Perftoran were followed for 3–5 years with no signs of adverse effects [6].

In preclinical studies, after 11 successive intraperitoneal infusions of Perftoran (total dose 275 mg/kg) to rats in the first stage of pregnancy, symptoms of teratogenicity were noted [1].

The primary side effects of Perftoran are mild to moderate hypersensitivity type symptoms, including temporary flushing or reddening of skin, increase in heart rate, decrease in blood pressure, rise in temperature, headache, substernal pain, dyspnea, neutropenia, and rarely an anaphylactic response. Typically, the symptoms are mild to moderate and resolve or improve within 5–15 min of the test dose, and the infusion is completed. If more serious symptoms arise, the infusion is stopped and symptoms are usually successfully treated with analgesics, sedatives, antipyretics, antihistamines, corticosteroids or adrenaline [5].

The reported incidence of these side effects is variable. In massive hemorrhage or trauma situations, the reported incidence is very low, possibly due to the inability to observe symptoms in obtunded patients. In cases where Perftoran is administered to awake patients, the incidence is variously reported as 1.8–4% [1], or 2–15% [5]. In a human clinical trial conducted in Mexico City in 2005, a test dose of 30 drops of Perftoran was administered to 16 patients prior to Cardiac surgery, and 1 patient (1/16, 6.25%) developed urticaria. The infusion was stopped and the patient was successfully treated with intravenous antihistamine. No adverse events were observed in 15 other patients receiving Perforan [4].

Perftoran is incompatible in the same infusion line with Dextrans, polyglucin, rheopolyglucin, hydroxyethyl starch, and oxyethyl amylumin infusions. It is compatible with blood and blood components, albumin, saline, most medications [5].

Per the Russian recommendations, contraindications to Perftoran include hemophilia, allergic diseases, and the first two trimesters of pregnancy. Relative contraindications include autoimmune diseases and history of hysteria [5].

Physiologic Properties

The primary physiologic property of interest is oxygen transport and delivery. As reviewed by Kaye in an earlier chapter in this book, PFC emulsions increase the oxygen carrying capacity of the plasma in proportion to their concentration in the plasma and the relative solubility of oxygen in the PFC components [9]. Perftoran emulsion is 10 volume per cent PFC, or 20% w/v PFC, and has an oxygen carrying capacity of 6.9 ml/dl of oxygen at PO_2 of 760 mmHg [1]. Compared to second generation PFC products such as Oxygent and Oxycyte, which contain up to 60% w/v PFC, Perftoran has significantly lower capacity for oxygen delivery, but, as explained by Spiess and others, the ability of PFC to facilitate oxygen movement through the plasma and extracellular fluids may be as important as bulk oxygen delivery capacity. The smaller particle size of Perftoran emulsion significantly increases overall surface area of the PFC particles and may improve oxygen diffusion through the plasma and tissues.

If given at its maximum recommended dose of 20 ml/kg in a 70 kg patient breathing an inspired oxygen concentration of 100%, 1.4 L of Perftoran would theoretically be able to carry 96.6 ml dissolved oxygen. In reality, the contribution of oxygen carried by Perftoran from the lungs to the tissues is probably less than the amount of oxygen carried and delivered by the hemoglobin in RBCs, even in relatively profound anemia. Of potentially greater importance is the ability of PFC to facilitate oxygen movement from the RBC through the plasma and tissue. Perftoran seems to provide the most benefit when given in lower doses in moderately severe anemia [1, 6].

Perftoran reportedly has a vasodilation effect, thought to be due to Nitric Oxide (NO) dissolution in RBCs [8]. It is also reported to have beneficial effects on the membrane of RBCs providing decreased rigidity and improved RBC survival [1, 6]. Perftoran has been shown to induce hepatic microsomal cytochrome P-450, which may be beneficial in treating certain toxicities [5, 8].

Pre-clinical Trials

Early animal trials were directed at establishing safety (mentioned above) and effectiveness as a treatment for severe hemorrhagic anemia. It is generally shown to be harmless and non-toxic to mice, rats, rabbits, and dogs.

A study in dogs with induced hemorrhagic shock by an acute blood loss of 35 ml/kg that were resuscitated after 1 h with 40 ml/kg Perftoran or Dextran 60 while breathing an oxygen air mixture. Two hours later, the kidneys were isolated and transplanted into anephric recipient dogs. The kid-

neys from the donor dogs treated with Perftoran showed improved tissue ATP/ADP, and decreased lactate/pyruvate ratios compared to the control group treated with Dextran 60. Recipient dogs in the Perftoran group showed lower levels of creatinine and urea, and renal graft survival was twice as long [1].

Other Animal Studies

Many exploratory animal studies have been performed with Perftoran for a variety of conditions. In Russia, Rat studies demonstrated that infusion of Perftoran 7.5 ml/kg compared to saline before moderate blood loss resulted in better preservation of mean arterial blood pressure (MAP). Hematopoiesis after massive blood replacement with Perftoran in rats was preserved [1]. Perftoran use in cardioplegic solutions delayed ischemic contracture and decrease in pH. Reperfusion of rabbit hearts after total ischemia with Perftoran provided a two-fold higher contractility amplitude compared to Tirode solution. Perftoran in doses exceeding 2 ml/kg was shown to induce cytochrome P450 synthesis and activate the monooxygenase system of the liver during the period of PFD retention. Perftoran was shown to promote earlier and more complete structural regeneration during reperfusion after partial or global critical intestinal ischemia [10]. In 2017 Perftoran was studied for resuscitation of cats after traumatic injury and it was demonstrated that oxygen saturation and tissue oxygenation was improved, and lactate production decreased over a recovery period of 7 days without adverse reactions [11].

In the USA, Eckman et al. in 2003 studied the clearance of microvascular gas embolism following Perftoran infusion in the rat cremaster muscle microcirculation. Perftoran infused before, but not after, gas embolization resulted in faster clearance of emboli and restoration of blood flow [12]. Abutarboush et al. in 2016 studied the effects of Perftoran on cerebral microcirculation and demonstrated that it did not cause additional vasoconstriction of cerebral pial arterioles or increase systemic blood pressure compared to saline or hetastarch [13].

Human Trials: Russia, Mexico

The detailed results of some of the clinical trials are in Russian references that are summarized and referenced in English language publications [1, 5, 6, 8]. This author cannot adequately review and reference the original trial results in Russian language publications and so must rely upon these English language summaries and refers the interested researcher to the reference lists of these publications for the original Russian references.

According to English language sources cited above, during preregistration clinical trials, Perftoran was given intravascularly to 964 patients for the following indications: Acute blood loss, hemorrhagic shock (22% of patients); Polytrauma, shock (20%); Toxic shock (20%); Limb ischemia (20.7%); Cardiac surgery (11.1%); Kidney transplantation (4.8%); Burns, oncology and other indications (8.2%) [1].

Dosage ranged from 4 to 30 ml/kg [6]. Trials involving Perftoran for blood loss anemia established the relative safety and side effect profile of Perftoran and demonstrated that it could facilitate oxygen delivery in blood loss anemia, improve hemodynamic parameters, and reduce the need for allogenic blood transfusion. Trials for other conditions reportedly demonstrated that Perftoran could alleviate symptoms of ischemia in vascular occlusive disease, improve recovery from polytrauma including traumatic brain injury, improve survival of organ transplants, and improve the function of hearts during cardiac bypass procedures. Additionally, they showed that Perftoran activates the detoxification functions of the liver, inhibits retro-virus infections, and is beneficial in local application to wounds and ulcers. Based on these trials, Perftoran was approved for registration in Russia and began to be sold in 1997 to Central Regional Stations of Blood Transfusion and some hospitals.

After registration approval, trials continued in a wider range of applications. A review of the Russian scientific literature from 1997 to 2002 included an additional 1823 patients that received Perftoran in comparative clinical trials with a total of 3332 patients. Some of these results are summarized by Maevsky et al. in 2005 Artificial Cells, Blood Substitutes and Biotechnology and in 2006 in the book Blood Substitutes edited by R. Winslow [1, 6]. A trial including 32 patients with gastroduodenal hemorrhage showed that Perftoran 900 ml given after control of bleeding with blood loss of 1500–2500 ml resulted in improved cardiac output and blood pressure, and enhancement of arterial and venous PO_2 compared to a control group of 30 patients [1]. A study including 39 patients receiving Peftoran during Gastroduodenal or colonic surgery with blood loss from 1000 to 2000 ml demonstrated that Perftoran augmented microcirculation in the liver, intestine, skeletal muscle and peritoneum by 15–30% [1]. A Perftoran dose recommendation for polytrauma adjusted the dose of Perftoran according to percent of volume blood loss from 2–4 ml/kg for 20% volume blood loss up to 10–15 ml/kg for volume blood loss exceeding 70%. They note improvements in PaO_2 and oxygen extraction, reduction in morbidity, and decrease in the use of donor blood [1, 6].

Other studies reported improvement of cerebral edema after traumatic brain injury, rapid recovery from coma due to fat or air embolism, and reduction of multi-organ dysfunction and respiratory distress syndrome [1]. Perftoran was

used for hemodilution during cardiac bypass in 45 patients and showed improvements in acidosis, lactate production, tissue oxygenation, blood viscosity, and erythrocyte survival compared to a control group of 60 patients receiving crystalloid hemodilution [1].

Multiple studies demonstrated benefit in limb ischemia. Skin PO_2 in ischemic limbs was improved by 30% after Perftoran 400 ml compared to only 6% with Dextran 40 infusion. Repeated Perftoran infusions decreased pain at rest and improved the distance that subjects could walk before pain onset for several months [6].

Several studies reported the reduction of necrotic region of the heart after myocardial infarction [6].

Post marketing surveys estimated that Perftoran had been administered to approximately 4500 patients by 2002 [6]. By 2016 it was estimated that Perftoran had been given to over 30,000 patients worldwide (Maslennikov I, Thompson D, July 2017, Scientific productive company Perftoran (Russia) and FluorO2 Therapeutics INC, Wake Forest NC, personal communication) [14].

A human clinical trial reported in the English language literature was conducted in Mexico City in 2004–2005 and reported in 2006 [4]. Perftoran was used in a trial of acute normovolemic hemodilution (ANH) during cardiac valvuloplasty with cardiopulmonary bypass. 15 patients were administered Perftoran during ANH and 15 control group patients received standard care with crystalloid and colloid solutions without Perftoran. Subjects receiving Perftoran maintained significantly higher intraoperative PaO_2 and pH and required fewer allogenic blood products than the control group. This trial is important because it provides objective data consistent with the Russian experience and showed no complications or deaths. One subject (1/16, 6.25%) developed urticaria after a small test dose of Perftoran which was easily managed.

Discussion

Perftoran has been shown to be a safe and moderately effective treatment in severe hemorrhagic anemia, and has been applied to multiple other conditions with promising results. While its lower concentration of PFC limits its oxygen carrying capacity in comparison to newer formulations such as Oxygent or Oxycyte, it may have advantages in overall side effect profile and tolerability. It is more stable and easier to store and prepare than Fluosol DA and reportedly has fewer side effects.

Perftoran has reportedly been administered to more humans than any other PFC preparation, with the possible exception of Fluosol DA. Adverse events have been well characterized and seem moderate and manageable. It is interesting to compare the side effect profile of Perftoran to the side effects of IV administration of the antibiotic Vancomycin, which include similar symptoms of flushing, hypotension, pruritis and occasional anaphylactoid reactions. Clinicians constantly weigh side effects against potential benefits of a drug or treatment. The question is always whether the potential benefit of a treatment is likely to be greater than the risks or side effects (including expense). For Perftoran, the weight of the evidence is reassuring that the risks and side effects are low to moderate and manageable, while the benefits are significant but highly variable, and sometimes speculative, depending upon the situation in which it is applied.

For hemorrhagic anemia, when applied appropriately, the evidence supports that Perftoran does improve tissue oxygenation, reduce acidosis, and facilitate a reduction in need for transfusion of red blood cells with a low risk of serious adverse effects. Whether these benefits are significant enough to justify use is dependent on many factors such as the availability and safety of the blood supply, and the wishes of the patient. It may be unlikely to gain widespread use in developed countries with robust blood banking capabilities, but could be more valuable in areas where the supply of safe blood products is more limited, or in situations where the need for blood products temporarily overwhelms the available supply, such as mass casualty situations or viral pandemics affecting the safety of the blood supply. It may also be useful for patients that object to blood products or for whom compatible blood is not available. As with other potential artificial oxygen carriers, controlled trials to prove superiority in comparison to blood products for acute hemorrhage are exceptionally difficult to conduct.

In conditions characterized by tissue ischemia, there is evidence from controlled animal experiments, limited human trials, and clinical experience, that Perftoran can provide at least temporary improvement in tissue oxygenation, but the side effect profile may limit application. It could provide a temporary bridge before or during more definitive correction of ischemia. For example, an ischemic limb might be temporarily supported during the time needed to correct a vascular occlusion or it could be used to temporarily improve brain tissue ischemia from embolic stroke in order to extend the time window for completion of endovascular thrombectomy. It is important to recognize that the only previously approved PFC product, Fluosol DA, was approved for relief of temporary ischemia during coronary angioplasty (not hemorrhagic anemia) [7].

There is renewed interest in the importance of tissue oxygenation in cancer therapy. Prior research has shown that susceptibility to radiation therapy and chemotherapy can be affected by the oxygenation of cancer cells [7] and it has recently been hypothesized that cancer immunotherapy might be improved by enhancing oxygen delivery to hypoxic areas of tumors [15]. Perftoran's extensive record of safety in a large number of human treatments, and its existing approval

in several countries, makes it a leading candidate for this application.

Animal studies with multiple PFC products, including Perftoran, have shown beneficial effects in the treatment of intravascular air or gas embolism, and decompression sickness [16–18]. It is nearly impossible to conduct a randomized controlled human trial for these rare but catastrophic events, but if Perftoran were to become available for another indication, it seems reasonable that it would be tried in cases where few other treatment options exist. An analogous situation is the use of intravenous lipid emulsion for severe local anesthetic toxicity ("Lipid rescue"), which has been used and widely endorsed despite never having a prospective or randomized trial and never receiving FDA approval for this use [19].

Conclusion

In this author's opinion, Perftoran failed to sustain commercial success for a variety of economic, and socio-political reasons, not solely for scientific or medical reasons. At the time of its introduction, there were significant efforts to develop competing PFC products with greater stability and concentration which seemed near to approval, so the commercial incentive to introduce or develop Perftoran in the Western Hemisphere was limited. Due to political restrictions on export, it was difficult for researchers in the USA to obtain "research grade" product, which limited the ability to replicate, confirm, or expand upon the research performed in Russia, and the standards for documentation of manufacturing processes were different in Russia compared to other parts of the world, which did not meet regulatory requirements for some markets. Availability and safety of blood products has improved worldwide, reducing the urgency of need for alternative treatments. Ultimately, the combination of these factors led to the suspension of manufacturing and sale of Perftoran, although it remains eligible for re-approval pending manufacture under GMP standards.

There are potential solutions to the problems described above. The competitive PFC formulations which initially seemed poised to be approved and marketed have all failed to be approved and their development is currently not proceeding forward in any significant manner (to this author's knowledge). Efforts are underway to move manufacturing of Perftoran to the USA under Good Manufacturing Practice certification, and there is renewed interest in Perftoran for several conditions which could provide a pathway to FDA approval, and it is eligible for approval in several countries pending GMP manufacturing. The track record that has already been established by administration to thousands of patients should be an advantage for efficiently verifying safety and effectiveness in other countries, although much of the data will inevitably need to be replicated and confirmed. In the final analysis, the available evidence seems to support the utility of Perftoran, but financial considerations will ultimately determine whether Perftoran will re-emerge as a commercially available product.

Key Points

- Perftoran is an intravenous perfluorocarbon emulsion developed and approved in Russia as an oxygen carrying plasma substitute for hemorrhagic anemia and other conditions.
- Perftoran has been administered to approximately 30,000 patients for a variety of indications and has been shown to enhance tissue oxygenation while having manageable side effects.
- The active components of Perftoran are similar to Fluosol DA which was developed in Japan and approved in the USA for use in coronary angioplasty and safely administered to thousands of patients. Perftoran reportedly has fewer side effects than Fluosol DA.
- There is abundant research data in Russian language literature. English language literature is relatively sparse and largely dependent upon Russian research. One human clinical trial in Mexico showed efficacy in cardiac surgery with acute normovolemic hemodilution and demonstrated a side effect profile consistent with Russian data.
- Perftoran is not currently available due to lack of GMP manufacturing certification during production in Russia. Plans are in development to resume production under GMP certification in USA.
- There is renewed interest in Perftoran for its historic indications as well as potential new indications in cancer immunotherapy, stroke, and vascular gas embolism.

Author's Disclosure Served as Consulting Chief Medical Officer of FluorO2 Therapeutics, INC., a pharmaceutical company intending to manufacture and market Perftoran under a new trade name (Vidaphor). This company has dissolved, but another company, Perftoran USA, LLC. Is being formed with the intention to manufacture Perftoran in the USA for research purposes and possibly for marketing in Mexico and other countries where it was previously approved. The author will have an equity stake in this company. The author may also have an equity or consulting relationship with Oxymmune Inc., a company researching the application of oxygen therapeutics to immunotherapy for cancer. Opinions expressed herein are solely the responsibility of the author.

It should be noted that most of the information herein is derived from literature from Russia or Russian scientists or corporations, and the author cannot independently verify the validity of all the information. The author has not personally participated in the cited research or administration of Perftoran to humans.

I would like to acknowledge the efforts of the late Deborah Thompson, co-founder of FluorO2 Therapeutics Inc. and a driving force in the development of several PFC products. Her goal was to bring at least one PFC emulsion product into availability for patients in need. I would also like to acknowledge the contributions of Igor Maslennikov of the Scientific-Productive Company Perftoran (Russia) and Perftoran USA LLC, and mentorship of Bruce D. Spiess, M.D.

References

1. Maevsky EI, Ivanitsky HR, Bogdanova L, Axenova O, Karmen NB, Pushkin SY, Maslennikov IA. Perftoran. In: Winslow RM, editor. Blood substitutes. London: Elsevier Academic Press; 2006. p. 288–97.
2. Maevsky EI. Emulsion of perfluoroorganic compounds for medical purposes, a process for the preparation thereof and methods for treating and preventing diseases with the use therof. United States Patent number 6,562,872 BI, 2003.
3. Vorobyev S. Perfluorocarbon gas transferring emulsion for medico-biological use, the composition and the production thereof a medicinal agent. European Patent Application number 06799621.5, 2006.
4. Verdin-Vasquez RC, Zapeda-Perez C, Ferra-Ferrer R, Chavez-Negrete A, Contreras F, Barroso-Aranda J. Use of perftoran emulsion to decrease allogenic blood transfusion in cardiac surgery: clinical trial. Artificial Cells Blood Substit Immobil Biotechnol. 2006;34:433–54.
5. Mayevskiy EI, Aksenova OG, Bogdanova LA, Moroz VV, SeninaRYa PSY, Ivanitsky GR. Analysis of clinical and adverse effects during phase I and II clinical studies of Perftoran. Russia Blood Bank Bull. 2001;4:2001.
6. Maevsky EI, Ivanitsky GR, Bogdanova LA, Axenova OG, Karmen NB, Zhiburt E, Senina RY, Pushkin SY, Maslennikov IA, Orlov A, Marinicheva I. Clinical results of Perftoran application: present and future. Artif Cells Blood Substit Immobil Biotechnol. 2005;33(1):37–46. https://doi.org/10.1081/BIO-200046654.
7. Lowe KC. Fluosol: The first commercial injectable perfluorocarbon oxygen carrier. In: Winslow RM, editor. Blood Substitutes. London, UK: Elsevier Academic Press; 2006. p. 288–97.
8. Gervits L, Maevsky EI. Perfluorocarbon-based blood substitute—Perftoran, Russian Experience. Chim Oggi/Chem Today. 2008;26(Suppl 3):Focus on Flourine Chemistry, 34–37.
9. Spiess BD. Perfluorocarbon emulsions as a promising technology: a review of tissue and vascular gas dynamics. J Appl Physiol. 2009;106(4):1444–52.
10. Kozhura VL, Basarab DA, Timkina MI, Golubev AM, Reshetnyak VI, Moroz VV. Reperfusion injury after critical intestinal ischemia and its correction with perfluorochemical emulsion "perftoran". World J Gastroenterol. 2005;11(45):7084–90.
11. Votrins SI, Vorobyev SI, Bolevich SB, Bolevich SS, Orlova AS, Tachieva BI, Korsakov DY, Nguyen MT, Sinelnikova TG, Novikov AA, Omarov IA. Use of perfluorocarbon based blood substitute Perftoran in correction of acute anemia in animals. Serb J Exp Clin Res. 2019;20:245–5o. https://doi.org/10.2478/SJECR-2018-0056.
12. Eckman DM, Lomivorotov VN. Microvascular gas embolization clearance following perfluorocarbon administration. J Appl Physiol. 2003;94:860–8. https://doi.org/10.1152/japplphysiol.00719.2002.
13. Abutarboush R, Saha BK, Mullah SH, Arnaud FG, Haque A, Aligbe C, Pappas G, Auker CR, McCarron RM, Moon-Massat PF, Scultetus AH. Cerebral microvascular and systemic effects following intravenous administration of perfluorocarbon emulsion Perftoran. J Funct Biomater. 2016;7:29. https://doi.org/10.3390/jfb7040029.
14. Latson GW. Perftoran (Vidaphor) – Introduction to western medicine. Shock J. 2019;52(1S Suppl 1):65–9. https://doi.org/10.1097/SHK.0000000000001063.
15. Hatfield SM, Sitkovsky M. Oxygenation to improve cancer vaccines, adoptive cell transfer and blockade of immunological negative regulators. Oncoimmunology. 2015;4(12):11–3. https://doi.org/10.1080/2162402X.2015.1052934.
16. Latson GW. Intravenous perfluorocarbon emulsions in the treatment of decompression sickness and arterial gas embolism – a review of evidence of therapeutic benefit. In: Moon RE, editor, Adjunctive therapy for decompression illness, report of the DCI adjunctive therapy Committee of the Undersea and Hyperbaric Medical Society, 2003.
17. Spiess BD, McCarthy RJ, Tuman KJ, Woronowicz AW, Tool KA, Ivankovich AD. Treatment of decompression sickness with a perfluorocarbon emulsion (FC-43). Undersea Biomed Res. 1988;15:31–7.
18. Spiess BD, Braverman B, Woronowicz AW, Ivankovich AD. Protection from cerebral air emboli with perfluorocarbons in rabbits. Stroke. 1986;17:1146–9.
19. American College of Medical Toxicology. ACMT position statement: interim guidance or the use of lipid resucitation therapy. J Med Toxicol. 2011;7:81–2. https://doi.org/10.1007/s13181-010-0125-3.

Oxycyte™

37

Richard T. Mahon

Introduction

Perhaps best known for his development of an Oxygen sensing electrode which bears his name (the Clark electrode), Leland C. Clark Jr. (1918–2005) had a robust investigative career relating directly to Oxycyte™ [1]. In 1966 Clark and Golan demonstrated that a mouse fully submerged in oxygenated perfluorcarbon (PFC) could sustain life [2]. Desiring to leverage the gas carrying properties of PFCs lead to his development of Oxycyte and forming of the company Synthetic Blood International (Costa Mesa CA) the 1990's. Synthetic Blood International now exists as Tenax Therapeutics (Morrisville NC), but had halted clinical development of Oxycyte. That task seems now to be left to Aurum Biosciences LTD (Glascow Scotland) under the development code name ABL-101 involving ischemic stroke imaging and therapy.

Sharing a common property of other PFCs; Flourinated-tert-butylcyclohexane (FTBC;$C_{10}F_{20}$) is both hydrophobic and lipophobic [3] and thus must be emulsified to be carried in blood.

The emulsified PFC thus being termed a perfluorocarbon-based Oxygen carrier (PFOC) Oxycyte™ (Tenax Therapeutics, Morrisville, NC) is an emulsification of 60% vol/vol FTBC with egg-yolk lethicin as the emulsifier and has an average particle size averages 200 nm with an Osmolality of 280mOSM [4].

The emulsification agent and the resultant particle size have significant effects on shelf-stability and intravascular stability of any PFOC [3, 5]. Though published data on these issues are not found, refrigerated shelf-life is measured in several months to a few years and blood half-life measured at about 18 h.[1] Once dissociated from the emulsion, as with other PFOCs, clearance is by exhalation of the volatilized compound and uptake by the reticuloendothelial system.

With all PFOCs, gases are carried by increased solubility based on Henry's law. At 760 torr FTBC has an Oxygen (O_2) solubility of 43 ml/100 ml and about 200 ml of Carbon Dioxide and 22 ml of Nitrogen [6]. This translates into Oxygen solubility of 0.017 $ml/dl_{Oxycyte}$ mmHg, being about six-fold higher than plasma at 0.00314 ml/dl_{plasma} mmHg [7].

Though Oxycyte increases O_2 carrying capacity based on the concentration present within blood (so called-Fluorocrit), its' ability to decrease diffusion resistance in *ex vivo and in vitro* models [8, 9] has been noted. Using an elegant closed peristaltic pump human blood system Torres Filho showed that the presence of Oxycyte (fluorcrit of 4%) enhanced O_2 extraction, suggesting that the normal resistance to O_2 flow from hemoglobin (Hb) was decreased, thus allowing Hb to be a more robust source of O_2 that can then be efficiently transported to tissue. Interestingly, this work was carried out with a PO_2 of 100 mmHg, suggesting this benefit does not require high partial pressure of inhaled O_2 [8]. Likewise, Cabrales showed that extreme hemodilution in hamsters was better tolerated with Oxycyte through increased O_2 extraction (though high partial pressures of O_2 were used) [9].

Another interesting property demonstrated with Oxycyte is surfactant like properties. In an endothelial cell culture model, Oxycyte inhibited the bubble induced mechano-transductional insult (Ca++ mediated) by negating adhesion forces between cell and bubble [10].

Recognition of the properties of decreased diffusion resistance and surfactant properties that informed many of the investigational and therapeutic trials of Oxycyte where the issue of "blood substitute" is minimized and the con-

R. T. Mahon (✉)
Undersea Medicine Department, Naval Medical Research Center, Silver Spring, MD, USA

[1]Personal communication Synthetic Blood International, Morrisville North Carolina while shelf stability studies were ongoing.

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_37

cept of O_2 therapeutics takes precedence. Disease states considered in Oxycyte research include anemia, central nervous system (CNS) disorders and bubble related conditions.

Anemia

Though the majority of current work with Oxycyte involves disease of tissue compromised by O_2 transport (as opposed to O_2 content) a few studies have evaluated Oxycyte in severe anemia and simulated hemorrhage.

Cabrales showed better tolerance of isovolumic hemodilution in rodents with respect to cardiac output and also showed enhanced O_2 extraction when Oxycyte was used during isovolemic hemodilution [9]. Also, in rodent isovolumic hemodilution, Oxycyte replacement improved O_2 content and maintained cerebral blood flow near baseline [11]. One can easily imagine the impact of improved transport of O_2 from Hb to tissue at risk for ischemia in treating several disease states.

Central Nervous System

Stroke

It is well recognized that decreased O_2 delivery to the tissue is, in part, responsible for significant decrements in delivery of O_2 to tissues. The central nervous system may be the best example of this. The decrease in blood flow with ischemic stroke leads to a cascade of energy deficiency, ionic imbalance, neurotransmitter dysfunction and ultimately cell death [12].

A benefit of improved O_2 flux would be anticipated in cerebral infarction. This was demonstrated in a rat model of permanent middle cerebral artery (MCA) occlusion, where Oxycyte (1 ml/100 g) was administrated directly after MCA occlusion. In that study gross infarction volume was improved when Oxycyte was combined with normobaric hyperoxia [13]. Perhaps this benefit was in preserving the penumbra as shown by Deuchar, demonstrating first that penumbra lactic acid was decreased following MCA occlusion (filament or embolic occlusion) in rats treated with Oxycyte [14] and later that Oxycyte combined with breathing a fraction of inspired O_2 (FiO_2) of 0.5 reduced infarct size and improved functional recovery (again rat MCA occlusion) [14]. Interestingly, Oxycyte, was used safely with tissue plasminogen activator (TPA) in experimental embolic stroke in rodents, setting the stage for clinical trials that can potentially change standard of care in stroke.

Traumatic Brain Injury

Traumatic brain injury (TBI) carries a significant individual and societal burden with more than 50 million cases world-wide per year. Severe TBI (a Glascow Coma scale of 3–8) carries a mortality of 30–40% with cerebral ischemic changes found in the majority of non-survivors [15]. Additionally, the time and duration of low brain tissue O_2 correlate with worse 6-month outcomes [16]. Interestingly, low brain tissue O_2 tension seems to be a function of diffusion impairment as opposed to O_2 delivery [17] again setting a plausible benefit for treatment with PFOC.

In a rodent lateral fluid percussion injury (LFPI) model of TBI, Oxygent combined with an FiO_2 of 1.0 improved brain O_2 tension in injured and uninjured animal over saline treated animals. Preservation of mitochondrial redox potential was also observed [18]. The same group then advanced these findings, but now using Oxycyte at 4.5 and 9 ml/kg along with FiO_2 1.0 O_2 to look at cognitive function as well as histologic signs of injury after LFPI. Both histologic injury and improved performance during Morris Water Maze testing were noted 15 days after injury [19].

A multicenter phase IIb study evaluating Oxycyte and TBI was halted in 2014. A review of Clinicaltrials.gov lists' start date in 2009 with termination (futility) in November 2014 and enrollment of 18 subjects. These results are not available in the literature.

Spinal Cord Injury

The acute phase of traumatic spinal cord injury involves mechanical disruption and compression of the spinal cord. This then results in a host of "secondary injuries" that in part can involve ischemia from direct disruption of vascular supply, local tissue edema as well as systemic hypotension and bradycardia (so called neurogenic shock) [20].

In rodents undergoing experimental contusive spinal cord injury (SCI), injured tissue PO_2 levels that fell dramatically, improved with enriched O_2 breathing gas and then further improved with Oxycyte administration [21]. This led to further experiments showing Oxycyte treated rodents after contusive SCI showed less white matter injury, smaller lesion area and less cell death [22]. In both of the above experiments Oxycyte was given 1 h after injury; a time frame that would be compatible with treating SCI in humans.

Bubble Related Diseases

Intravascular bubbles can result from significant changes in ambient pressure (decompression sickness), iatrogenic introduction (air gas embolism) and as a complication of disease

such as asthma in exacerbation [23]. Though a pathophysiologic oversimplification, intravascular bubbles can obstruct blood flow, cause inflammation and induce vasoconstriction. All of which can lead to tissue injury from ischemia. The innate gas dissolving properties of PFC combined with the small particle size of PFOC set the stage for research in bubble related diseases.

Air Gas Embolism (AGE)

Air (or other gas) can enter the vascular system during a myriad of procedures, either directly into the venous or arterial system or even as part of a complication of disease states [23]. When entering the venous system, the robust capillary bed of the lung can serve as an effective filter [24] but that can be overwhelmed or even bypassed by intracardiac shunts [25].

Venous air can lead to acute right heart failure or become arterialized by right-to-left heart shunting. Once arterialized, bubbles can serve to have mechanical, embolic and biochemical effects that can be trivial, cause significant impairment or prove fatal [26].

Though the mechanical obstructing effects of bubble are easy to imagine the biochemical effects are less so.

In vitro work has shown that in human umbilical vein endothelial cells (HUVEC) bubble contact caused an intracellular calcium upsurge that was in part mediated by the glycocalyx. Adding Oxycyte in the role of a "surface active perfluorcarbon" significantly abolished this calcium influx [10]. *In vivo* showed that pre-treating with Oxycyte (10 volume percent blood) prior to experimental cerebral gas embolism in a rodent model negated cerebral damage as measured by MRI (as did the pure surfactanct PF-127) and preserved functional capacity (as measured by preserved time to latency in Morris Water Maze - all while breathing an FiO_2 of 0.3) [27].

AGE commonly is associated with critical illness and the standard treatment is hyperbaric O_2 (HBO). The lack of HBO availability in many centers and the challenge of transporting critically ill patients commonly makes AGE treatment problematic. Further testing of Oxycyte in AGE holds promise for enhancing care and improving outcomes in this difficult clinical scenario.

Decompression Sickness (DCS)

Gas in tissue is absorbed based on the solubility coefficient of that tissue and the partial pressure of that gas as determined by ambient pressure (Henry's law). As such, a decrease in ambient pressure decreases the amount of tissue gas that can be solubilized. This state is known as supersaturation. Although all gases are subject to these laws, it is generally "inert gases" (such as Nitrogen) that are most abundant and involved in the pathophysiology of decompression sickness (DCS).

Supersaturation of tissue can then lead to bubble formation manifesting in tissue or in the vasculature. This can result in several abnormalities including mechanical obstruction of blood flow, arterial constriction, impaired venous drainage and multiple biochemical abnormalities leading to the syndromic disease of decompression sickness (DCS) [26].

Divers (and other hyperbaric workers) are at risk for DCS because as they are exposed to increase ambient pressure and thus increase tissue gas content that needs to be dealt with when returning to normal surface pressure. DCS can also be seen in low pressure environments (high altitude flight and space extravehicular activities) where the gas content of tissue that is normally present during existence at "surface pressure" leads to supersaturation when ambient pressure is significantly reduced.

Clinically DCS can manifest with "minor" joint aches, skin "marbling" or "major" complications such as neurologic injury or cardiopulmonary collapse ("chokes"). Of the "major" manifestations of DCS, spinal cord injury may result in permanent compromise of function with residual symptoms after treatment in 30% of subjects [28]. Cardiopulmonary manifestations have a high risk of death.

In general, DCS is treated with supplemental O_2 to (enhance the elimination of accumulated "inert gas" and recompression therapy that serves in part to decrease the size of intravascular bubbles (Boyles Law)). Recompression typically is accomplished by using a hyperbaric chamber capable of elevating ambient pressure. Such chambers are costly in equipment and staffing and not widely available. These constraints poses problems in remote locations, which can be compounded by mass casualty situations as may be seen in disabled submarine accidents [29].

With the mechanisms of DCS surrounding elevation in whole body inert gas content and the adverse effects of bubbles; a compound that would enhance inert gas elimination, enhance O_2 delivery to tissues compromised by bubble obstructed blood flow, and mitigate the biochemical consequences of intravascular bubbles would be of great interest for the treatment of DCS. Such "non-recompressive" treatment has the potential to enhance DCS management in remote locations or in mass casualty scenarios such as disabled submarine.

PFOCs have been studied for the treatment of DCS ince the 1980's [6],with several products having been studied in animal models with various dosing, timing of dose and outcome measures. In the quest for non-recompressive therapies, Oxycyte is unique in having the most robust set of large animal studies of all PFOCs.

Cardiopulmonary DCS manifests as cardiovascular collapse resulting from an overwhelming amount of intravascular bubbles causing acute elevation in pulmonary artery pressure and hence right heart failure [30].

The group at Naval Medical Research Center (Silver Spring Maryland), embarked on a series of studies evaluating endpoints of survival and spinal cord injury in large animal models of severe DCS. In a swine model Oxycyte at 5 ml/kg conferred a survival benefit in severe DCS and improved spinal cord injury at 3 cc/kg (but not mortality) [31, 32]. Oxycyte also was associated with less spinal cord injury from severe DCS in an Ovine model using an Oxycyte dose of 5 mg/kg [33].

Oxycyte Safety in DCS

In animal studies Oxycyte seems beneficial for treating severe DCS, but a question about its safety remains. Though Oxycyte appears beneficial in treating DCS (animal models), it may not be curative. Hence there may be a need for recompression therapy in subjects with ongoing symptoms. One feared complication of recompression therapy with HBO is seizures, termed central nervous system oxygen toxicity (CNSOT).

Central Nervous System Oxygen Toxicity

HBO is associated with cerebral vasoconstriction and lower cerebral blood flow (CBF). CNSOT is likely related to free radical generation from HBO resulting in a pathologic increase in CBF. As PFOC all increase blood O_2 content, the concern for increased CNSOT with HBO is logical.

In a rodent of model of CNS-OT, Oxycyte (6 ml/kg) was associated with increased regional CNS PO_2 as well as decreased time to seizure at 5 times atmospheric pressure (5 ATA) [34]. Of note in that work a significantly decrease in time to seizure was not observed using a lower dose of Oxycyte (3 ml/kg) undergoing the same exposure. However, in a swine model CNSOT, Oxycyte at a dose of 5 cc/kg did not decrease time to seizure latency with exposure to 6 ATA O_2 and importantly had no change in seizure resolution time when compared to control animals [35].

The effect of Oxycyte on seizure risk in swine was further tested in a mixed gender swine mode of DCS treatment using Oxycyte at 4 cc/kg followed in 4 h by standard recompression therapy at 2.8 ATA O_2 following a standard Navy Treatment Table 6 which resulted in no seizure (control or Oxycyte group) [36]. However, there was no overall mortality benefit and a statistically non-significant signal suggested perhaps an increased mortality in female swine treated with Oxycyte [36].

Pulmonary Artery Pressure (PAP)

An elevated pulmonary artery pressure (PAP) in *Artiadactyla* (swine, sheep, goats, etc.) without DCS has been described with PFOC and has been attributed to a similarly transient PAP rise with other small particulate injections [37]. This is likely related to a complement activated pseudo allergy (CARPA), with one major driver of CARPA being particle size [38].

With the known association of PAP elevation considered, the effects of the PFOC Oxygent (Alliance Pharmaceutical, San Diego CA) at 4.5 ml/kg on PAP in juvenile swine exposed to an arduous compression-decompression profile was studied. Though Oxygent was shown to elevated PAP in control animals (no compression-decompression), the elevated in PAP after decompression was not different between Oxygent and saline control animals [39].

Oxycyte was evaluated in a similar fashion in a swine model of severe DCS. Severe DCS in this model was associated with elevated PAP and the administration of Oxycyte lead to further increase in PAP that appeared similar between male and female swine. Of note the manifestations of severe DCS were quicker and more severe in females irrelevant to treatment with or without Oxycyte [40]. With respect to humans, there appears no evidence of similar concerns in humans administered Oxycyte in the literature.

Hematologic

Part of the biochemical consequences of DCS can be thrombocytopenia [41]. Thrombocytopenia has also been noted with administration of other PFOCs [42].

With the obvious concern for the interaction of DCS and PFOC, the combined interaction with DCS and Oxycyte was studied in a swine model. In that work no bleeding occurred, and platelet counts were not different in Oxycyte versus saline control animals studied over 8 days of analysis. However, there was a significant change in prothrombin time and activated partial thromboplastin time [43].

Hence, Oxycyte appears beneficial in treating DCS and has reasonable evidence of safety in animal models. For human use, safety studies will need to proceed before therapeutic clinical trials are performed. Such studies would be logistically challenging, but feasible and could fill a gap in treating DCS, especially in disabled submarine incidents.

Summary

Forty years after Fluosal DA (GreenCross Japan) was developed as a PFOC, a greater understanding of PFOC O_2 transport, delivery and surfactant properties has been gained. Additionally, technology has allowed higher PFC content,

smaller emulsified particle size and enhanced shelf and intravital stability. Oxycyte is well studied in animal models and shows promising therapeutic benefit in several disease states. Transition to human subject research has been lackluster, yet hope remains that future research Oxycyte studies will lead to improved treatment for some of the devastating diseases reviewed here.

The views expressed in this article do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the United States Government.

Key Points

- Oxycyte increases Oxygen carrying capacity and enhances diffusion of Oxygen to tissues.
- Oxycyte shows benefit in in several animal models involving stoke, traumatic brain injury, air gas embolism and decompression sickness
- Bubble-related disease treatment appears to benefit from surfactant type properties of Oxycyte
- Oxycyte is the most studied compound in large animal models with decompression sickness
- The safety profile of Oxycyte treatment in decompression sickness appears favorable in animal models.

References

1. Heineman WR, Jensen WB. Obituary: Leland C. Clark Jr (1918-2005). Biosens Bioelectron. 2006;8(21):1403–4.
2. Clark LC, Golan F. Survival of mammals breathing organic liquids equilibrated with oxygen at atmospheric pressure. Science. 1966;152:1755–6.
3. Jägers J, Wrobeln A, Ferenz KB. Perfluorocarbon-based oxygen carriers: from physics to physiology. Pflugers Arch - Eur J Physiol. 2021;473:139–50. https://doi.org/10.1007/s00424-020-02482-2.
4. Torres LN, Spiess BD, Torres Filho IP. Effects of perfluorocarbon emulsions on microvascular blood flow and oxygen transport in a model of severe arterial gas embolism. J Surg Res. 2014;187(1):324–33. https://doi.org/10.1016/j.jss.2013.08.011. Epub 2013 Sep 21. PMID: 24246440.
5. Okamoto H, Yamanouchi K, Yokoyama K. Retention of perfluorochemicals in circulating blood and organs of animals after intravenous injection of their emulsions. Chem Pharm Bull (Tokyo). 1975;23(7):1452–7. https://doi.org/10.1248/cpb.23.1452. PMID: 1182885.
6. Spiess BD. Perfluorocarbon emulsions as a promising technology: a review of tissue and vascular gas dynamics. J Appl Physiol. 2009;106(4):1444–52. https://doi.org/10.1152/japplphysiol.90995.2008. Epub 2009 Jan 29. PMID: 19179651.
7. Castro CI, Briceno JC. Perfluorocarbon-based oxygen carriers: review of products and trials. Artif Organs. 2010;34(8):622–34. https://doi.org/10.1111/j.1525-1594.2009.00944.x. PMID: 20698841.
8. Torres Filho IP, Pedro JR, Narayanan SV, Nguyen NM, Roseff SD, Spiess BD. Perfluorocarbon emulsion improves oxygen transport of normal and sickle cell human blood in vitro. J Biomed Mater Res A. 2014;102(7):2105–15. https://doi.org/10.1002/jbm.a.34885. Epub 2013 Aug 2. PMID: 23894124.
9. Cabrales P, Carlos BJ. Delaying blood transfusion in experimental acute anemia with a perfluorocarbon emulsion. Anesthesiology. 2011;114(4):901–11. https://doi.org/10.1097/ALN.0b013e31820efb36. PMID: 21326091; PMCID: PMC3063325.
10. Klinger AL, Pichette B, Sobolewski P, Eckmann DM. Mechanotransductional basis of endothelial cell response to intravascular bubbles. Integr Biol (Camb). 2011;3(10):1033–42. https://doi.org/10.1039/c1ib00017a. Epub 2011 Sep 19. PMID: 21931900; PMCID: PMC3186877.
11. Yang ZJ, Price CD, Bosco G, Tucci M, El-Badri NS, Mangar D, Camporesi EM. The effect of isovolemic hemodilution with oxycyte, a perfluorocarbon emulsion, on cerebral blood flow in rats. PLoS One. 2008;3(4):e2010. https://doi.org/10.1371/journal.pone.0002010. PMID: 18431491; PMCID: PMC2291566.
12. Xing C, Arai K, Lo EH, Hommel M. Pathophysiologic cascades in ischemic stroke. Int J Stroke. 2012;7(5):378–85. https://doi.org/10.1111/j.1747-4949.2012.00839.x. PMID: 22712739; PMCID: PMC3985770.
13. Woitzik J, Weinzierl N, Schilling L. Early administration of a second-generation perfluorochemical decreases ischemic brain damage in a model of permanent middle cerebral artery occlusion in the rat. Neurol Res. 2005;27(5):509–15. https://doi.org/10.1179/016164105X15677. PMID: 15978177.
14. Deuchar GA, van Kralingen JC, Work LM, Santosh C, Muir KW, McCabe C, Macrae IM. Preclinical validation of the therapeutic potential of Glasgow oxygen level dependent (GOLD) technology: a theranostic for acute stroke. Transl Stroke Res. 2019;10(5):583–95. https://doi.org/10.1007/s12975-018-0679-y. Epub 2018 Nov 30. PMID: 30506268; PMCID: PMC6733820.
15. Khellaf A, Khan DZ, Helmy A. Recent advances in traumatic brain injury. J Neurol. 2019;266(11):2878–89. https://doi.org/10.1007/s00415-019-09541-4. Epub 2019 Sep 28. PMID: 31563989; PMCID: PMC6803592.
16. van den Brink WA, van Santbrink H, Steyerberg EW, Avezaat CJ, Suazo JA, Hogesteeger C, Jansen WJ, Kloos LM, Vermeulen J, Maas AI. Brain oxygen tension in severe head injury. Neurosurgery. 2000;46(4):868–76; discussion 876–8. https://doi.org/10.1097/00006123-200004000-00018. PMID: 10764260.
17. Rosenthal G, Hemphill JC 3rd, Sorani M, Martin C, Morabito D, Obrist WD, Manley GT. Brain tissue oxygen tension is more indicative of oxygen diffusion than oxygen delivery and metabolism in patients with traumatic brain injury. Crit Care Med. 2008;36(6):1917–24. https://doi.org/10.1097/CCM.0b013e3181743d77. PMID: 18496376.
18. Daugherty WP, Levasseur JE, Sun D, Spiess BD, Bullock MR. Perfluorocarbon emulsion improves cerebral oxygenation and mitochondrial function after fluid percussion brain injury in rats. Neurosurgery. 2004;54(5):1223–30; discussion 1230. https://doi.org/10.1227/01.neu.0000119238.68938.5d. PMID: 15113478.
19. Zhou Z, Sun D, Levasseur JE, Merenda A, Hamm RJ, Zhu J, Spiess BD, Bullock MR. Perfluorocarbon emulsions improve cognitive recovery after lateral fluid percussion brain injury in rats. Neurosurgery. 2008;63(4):799–806; discussion 806–7. https://doi.org/10.1227/01.NEU.0000325493.51900.53. PMID: 18981892.
20. Anjum A, Yazid MD, Fauzi Daud M, Idris J, Ng AMH, Selvi Naicker A, Ismail OHR, Athi Kumar RK, Lokanathan Y. Spinal cord injury: pathophysiology, multimolecular interactions, and underlying recovery mechanisms. Int J Mol Sci. 2020;21(20):7533. https://doi.org/10.3390/ijms21207533. PMID: 33066029; PMCID: PMC7589539.
21. Schroeder JL, Highsmith JM, Young HF, Mathern BE. Reduction of hypoxia by perfluorocarbon emulsion in a traumatic spinal cord injury model. J Neurosurg Spine. 2008;9(2):213–20. https://doi.org/10.3171/SPI/2008/9/8/213. PMID: 18764757.

22. Yacoub A, Hajec MC, Stanger R, Wan W, Young H, Mathern BE. Neuroprotective effects of perflurocarbon (oxycyte) after contusive spinal cord injury. J Neurotrauma. 2014;31(3):256–67. https://doi.org/10.1089/neu.2013.3037. Epub 2013 Nov 21. PMID: 24025081; PMCID: PMC3904515.
23. Moon RE. Gas embolism. In: Oriani G, Marroni A, Wattel F, editors. Handbook on hyperbaric medicine. Milano: Springer; 1996. https://doi.org/10.1007/978-88-470-2198-3.
24. Butler BD, Robinson R, Little T, Chelly JE, Doursout MF. Cardiopulmonary changes with moderate decompression in rats. Undersea Hyperb Med. 1996;23(2):83–9. PMID: 8840476.
25. Wilmshurst P. Risk mitigation in divers with persistent (patent) foramen ovale. Diving Hyperb Med. 2019;49(2):77–8. https://doi.org/10.28920/dhm49.2.77-78. PMID: 31177512; PMCID: PMC6704006.
26. Vann RD, Butler FK, Mitchell SJ, Moon RE. Decompression illness. Lancet. 2011;377(9760):153–64. https://doi.org/10.1016/S0140-6736(10)61085-9. PMID: 21215883.
27. Eckmann DM, Armstead SC. Surfactant reduction of cerebral infarct size and behavioral deficit in a rat model of cerebrovascular arterial gas embolism. J Appl Physiol (1985). 2013;115(6):868–76. https://doi.org/10.1152/japplphysiol.01382.2012. Epub 2013 July 11. PMID: 23845977; PMCID: PMC3764619.
28. Blatteau JE, Gempp E, Simon O, Coulange M, Delafosse B, Souday V, Cochard G, Arvieux J, Henckes A, Lafere P, Germonpre P, Lapoussiere JM, Hugon M, Constantin P, Barthelemy A. Prognostic factors of spinal cord decompression sickness in recreational diving: retrospective and multicentric analysis of 279 cases. Neurocrit Care. 2011;15(1):120–7. https://doi.org/10.1007/s12028-010-9370-1. PMID: 20734244.
29. Parker EC, Ball R, Tibbles PM, Weathersby PK. Escape from a disabled submarine: decompression sickness risk estimation. Aviat Space Environ Med. 2000;71(2):109–14. PMID: 10685582.
30. Catron PW, Flynn ET Jr, Yaffe L, Bradley ME, Thomas LB, Hinman D, Survanshi S, Johnson JT, Harrington J. Morphological and physiological responses of the lungs of dogs to acute decompression. J Appl Physiol Respir Environ Exerc Physiol. 1984;57(2):467–74. https://doi.org/10.1152/jappl.1984.57.2.467. PMID: 6469817.
31. Mahon RT, Watanabe TT, Wilson MC, Auker CR. Intravenous perfluorocarbon after onset of decompression sickness decreases mortality in 20-kg swine. Aviat Space Environ Med. 2010;81(6):555–9. https://doi.org/10.3357/asem.2745.2010. PMID: 20540446.
32. Mahon RT, Auker CR, Bradley SG, Mendelson A, Hall AA. The emulsified perfluorocarbon Oxycyte improves spinal cord injury in a swine model of decompression sickness. Spinal Cord. 2013;51(3):188–92. https://doi.org/10.1038/sc.2012.135. Epub 2012 Nov 20. PMID: 23165506.
33. Cronin WA, Khan K, Hall AA, Bodo M, Mahon RT. The effect of the perfluorocarbon emulsion Oxycyte™ in an ovine model of severe decompression illness. Undersea Hyperb Med. 2021;48(1):25–31. PMID: 33648030.
34. Demchenko IT, Mahon RT, Allen BW, Piantadosi CA. Brain oxygenation and CNS oxygen toxicity after infusion of perfluorocarbon emulsion. J Appl Physiol (1985). 2012;113(2):224–31. https://doi.org/10.1152/japplphysiol.00308.2012. Epub 2012 May 3. PMID: 22556400.
35. Mahon RT, Hall A, Bodo M, Auker C. The intravenous perfluorocarbon emulsion Oxycyte does not increase hyperbaric oxygen-related seizures in a non-sedated swine model. Eur J Appl Physiol. 2013;113(11):2795–802. https://doi.org/10.1007/s00421-013-2720-x. Epub 2013 Sep 6. PMID: 24062008.
36. Cronin WA, Hall AA, Auker CR, Mahon RT. Perfluorocarbon in delayed recompression with a mixed gender swine model of decompression sickness. Aerosp Med Hum Perform. 2018;89(1):14–8. https://doi.org/10.3357/AMHP.4925.2018. PMID: 29233239.
37. Flaim SF. Pharmacokinetics and side effects of perfluorocarbon-based blood substitutes. Artif Cells Blood Substit Immobil Biotechnol. 1994;22(4):1043–54. https://doi.org/10.3109/10731199409138801. PMID: 7849908.
38. Szebeni J, Baranyi L, Savay S, Bodo M, Morse DS, Basta M, Stahl GL, Bünger R, Alving CR. Liposome-induced pulmonary hypertension: properties and mechanism of a complement-mediated pseudoallergic reaction. Am J Physiol Heart Circ Physiol. 2000;279(3):H1319–28. https://doi.org/10.1152/ajpheart.2000.279.3.H1319. PMID: 10993799.
39. Spiess BD, Zhu J, Pierce B, Weis R, Berger BE, Reses J, Smith CR, Ewbank B, Ward KR. Effects of perfluorocarbon infusion in an anesthetized swine decompression model. J Surg Res. 2009;153(1):83–94. https://doi.org/10.1016/j.jss.2008.02.045. Epub 2008 Mar 26. PMID: 18541265.
40. Mahon RT, Cronin WA, Bodo M, Tirumala S, Regis DP, Auker CR. Cardiovascular parameters in a mixed-sex swine study of severe decompression sickness treated with the emulsified perfluorocarbon Oxycyte. J Appl Physiol (1985). 2015;118(1):71–9. https://doi.org/10.1152/japplphysiol.00727.2014. Epub 2014 Oct 23. PMID: 25342702.
41. Goad RF, Neuman TS, Linaweaver PG Jr. Hematologic changes in man during decompression: relations to overt decompression sickness and bubble scores. Aviat Space Environ Med. 1976;47(8):863–7. PMID: 949307.
42. Spahn DR. Blood substitutes. Artificial oxygen carriers: perfluorocarbon emulsions. Crit Care. 1999;3(5):R93–7. https://doi.org/10.1186/cc364. Epub 1999 Sep 24. PMID: 11094488; PMCID: PMC137239.
43. Cronin WA, Senese AL, Arnaud FG, Regis DP, Auker CR, Mahon RT. The effect of the perfluorocarbon emulsion Oxycyte on platelet count and function in the treatment of decompression sickness in a swine model. Blood Coagul Fibrinolysis. 2016;27(6):702–10. https://doi.org/10.1097/MBC.0000000000000481. PMID: 26650458.

Hemoximer: History, Pharmacology, Pre-Clinical Studies, Clinical Trials, and Lessons Learned

38

Christopher Priavalle and Joe De Angelo

Introduction

Pyridoxalated hemoglobin-polyoxyethylene conjugate (*PHP/hemoximer*) is prepared from stroma-free human hemoglobin (SFH), chemically modified with pyridoxal-5'-phosphate, and coupled with activated polyoxyethylene conjugate ester to yield *PHP* [1].

Apex Bioscience, Inc. proposed that this new compound be developed as a vasopressor for hypotension refractory to standard therapy from a variety of etiologies, such as sepsis, interleukin-2 induced hypotension, and hemodialysis-induced hypotension. Preclinical evidence demonstrated that PHP exhibited the desired physiological effects of increasing vascular tone, thereby leading to an increase in blood pressure. Based on preliminary animal studies, it was estimated that the therapeutic dose for the treatment of hypotension in the population with refractory shock secondary to sepsis would range between 25 and 100 mg Hb/kg body weight.

The effectiveness of PHP in raising blood pressure was postulated to occur or was mediated by "scavenging" the excess levels of nitric oxide (NO) produced in the vasculature. The role of NO in a variety of shock states had been extensively studied [2] and a review summarized its role in shock associated with systemic inflammatory response syndrome [3]. NO had been demonstrated to be the primary effector in endotoxin (LPS)-induced hypotension associated with sepsis or presumed sepsis [4]. Increased NO levels had also been associated with hypotension screening during hemodialysis [5] and with various forms of heart failure [6]. In addition to its vasoactivity, NO had also been shown to be a myocardial depressant [7, 8], an inhibitor of mitochondrial electron transport [9], an inducer of vascular leakage [10], and an enhancer of LPS-induced cytokine release [11]. Hence, it was postulated to be involved in a wide variety of shock etiologies.

NO metabolites such as nitrosyl-hemoglobin had been shown to accumulate in animal models of septic shock [12–14]. NO production had also been correlated in human septic shock with increased cardiac output [15], decreased systemic vascular resistance [15, 16], and increased disease severity [15]. Reduction of NO levels had been proposed as a therapy for septic shock [3], and in initial human studies patients suffering from refractory shock secondary to sepsis would be the target population.

The biochemistry of hemoglobin (Hb) interactions with nitric oxide had been extensively studied [17–25]. Hemoglobin has an extremely high affinity for NO -about 200,000 times greater than the affinity of Hb for oxygen [20]. The on-rate for NO is extremely fast - on the order of 10^7 M-1 sec-1 [17]. In addition to rapid binding of NO by Hb, oxyhemoglobin oxidizes NO to nitrate in an equally rapid reaction [19]. This is thought to be the major route of NO destruction in vivo, accounting for as much as 96% of NO catabolism [25]. Without this rapid destruction of NO by RBC hemoglobin, NO would likely lose its ability to act locally and be unable to function as an autocrine and paracrine effector.

Cell-free hemoglobins, under development as blood substitutes, have been shown to have vasopressor activity in humans [26]. These effects have been shown to be mediated by hemoglobin-inactivation of NO [27–29]. Studies have also been conducted showing the ability of Hb to reverse LPS-induced vasodilation in vascular rings and to restore normal responsivity in pressor-refractory vessels [30]. Nitric oxide synthase inhibitors have been used in human septic shock and have been shown to reverse acute hypotension [31]. It was therefore proposed that NO scavengers, such as PHP, would be equally efficacious in this indication.

Both in vitro and in vivo studies demonstrated the effectiveness of PHP in enhancing vascular tone and raising blood pressure of animals through an NO-dependent mechanism [38]. Enhancement of vascular tone was shown in vitro using

C. Priavalle
Apex Bioscience, Inc., Durham, NC, USA

J. De Angelo (✉)
Platelet Therapeutics, LLC, Chapel Hill, NC, USA
e-mail: jdeangelo@alum.mit.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_38

dog basilar arteries, which were constricted by low concentrations of PHP. Both stroma-free hemoglobin and PHP prevented the vascular relaxation induced by arginine, an NO precursor, and acetylcholine, which acts through the release of NO. Studies in vivo using rats had shown that PHP significantly raises blood pressure at doses less than 100 mg/kg. The blood pressure responses were not significantly antagonized by antiadrenergic or cyclooxygenase inhibitors. However, elevation of blood pressure by PHP was reduced by the administration of the NO synthase inhibitor, NG-monomethyl-L-arginine, which lowers available vasoactive NO [29].

PHP has been shown to be associated with restoration of blood pressure in a sheep model of hyperdynamic sepsis at doses as low as 50 mg Hb/kg. In addition, PHP has been shown to restore vascular responsiveness of rat aortic rings to the vasopressor phenylephrine after incubation with endotoxin (LPS).

Chemistry and Physical Properties

See Fig. 38.1 for Hemoximer structural formula and characteristics.

See Table 38.1 for Characteristics of hemoximer.

C. **Safety**

PHP is made from stroma-free hemoglobin (SFH) purified from outdated human red blood cells. The blood used in all manufacturing operations has been screened using FDA-licensed tests. All units used are negative for antibodies to HIV, hepatitis B core antigen, human T-cell lymphotropic virus, type I and hepatitis C. The units are also negative for hepatitis B core antigen and the level of alanine aminotransferase is within acceptable limits.

Fig. 38.1 Chemistry and physical properties, structural formula and characteristics

Structural Formula
$(PLP)_nHb\ (POE)_m$
PLP = Pyridoxal-5′-phosphate
Hb = Human hemoglobin
POE = Polyoxyethylene (□-carboxymethyl-□-carboxymethoxypolyoxyethylene)
POE = $HO_2CCH_2(OCH_2CH_2)_{62}OCH_2CO_2H$, average molecular weight 3000
n = 3.3 ± 0.1, m = 5.0 ± 0.4
Characteristics
Appearance
Clear, deep red solution containing 8 g/dL of *PHP*on a hemoglobin basis.
Colloid Osmotic Pressure (Oncotic Pressure)
At 8 g/dL the oncotic pressure of *PHP*is approximately 49 mm Hg.
Viscosity
At 8 g/dL the viscosity of *PHP* is approximately 3 centipoise (whole blood is 4.2 centipoise)

Table 38.1 Characteristics of hemoximer (average of 3 typical production lots)

Characteristic	Value
Hemoglobin concentration	8 g/dL
Methemoglobin content	1.9%
pH	7.4
Molecular weight	109,000
Osmolality	270 mmol/kg
P50	23.4 mm Hg
Pyrogenicity	Non-pyrogenic
Sterility	Sterile
General safety	Safe in Guinea Pigs and Mice
Phosphatidylethanolamine content	≤0.25 ppm
Endotoxin content	0.11 EU/mL

In addition, the production process used to produce *PHP* has been demonstrated to be capable of removal and inactivation of adventitious agents. Distinct, orthogonal steps for removal of enveloped and non-enveloped viruses and inactivation of enveloped viruses are performed during the *PHP* production process. Studies have shown the process can remove and/or inactivate HIV, bovine viral diarrhea virus (as a model for hepatitis C), and hepatitis A.

PHP is a sterile product. The product contains no blood group antigens and does not require typing prior to administration. Stroma components from the red blood cells are removed by the production process.

Non-clinical Studies

Early studies of PHP were conducted by Ajinomoto in models of hemorrhagic shock [30–39]. The chemical and biochemical properties of PHP, in particular, its antioxidant properties were examined in detail [40–54].

Extensive studies were conducted in a hyperdynamic sepsis model induced in sheep by infusion of live bacteria [55–64]. In the septic sheep model, PHP normalized mean arterial pressure to pre-sepsis levels, increased systemic vascular resistance, reduced the dose of norepinephrine needed to increase blood pressure, and tended to improve myocardial contractility. Hypoxic pulmonary vasoconstriction was not affected by PHP. Regional blood flow was unchanged. PHP infusion increased renal glomerular filtration rate and urinary output. The results of these studies were consistent with the action of PHP as an NO scavenger and suggested potential benefit as a treatment for distributive shock related to sepsis.

Clinical Studies

Phase 1

The initial Phase 1 study [unpublished results] was a double-blind, placebo-controlled trial in 18 healthy volunteers to assess safety and tolerance to a range of PHP doses (25, 50, and 100 mg/kg) given by intravenous infusion over 30 min. The endpoints were clinical evaluation, laboratory tests and pharmacokinetics. The trial was completed in November 1995, see Table 38.2 for results.

Table 38.2 Hemoximer phase 1 study results

PHP treatment showed:
No significant adverse events
No significant changes in blood pressure
No significant gastrointestinal symptoms
No significant coagulation abnormalities
No significant effect on hematological parameters

Phase 2a

The Phase 2a trial [unpublished results] was an open label study in 18 vasopressor-dependent septic shock patients to establish the appropriate dose and schedule for future pivotal trials. The dose range (25, 50, and 100 mg/kg) of PHP was given by a single intravenous bolus infusion over 30 min. The primary endpoints were safety, tolerance, and blood pressure effects. The secondary endpoints were vasopressor use and pharmacokinetics.

- All three doses of PHP increased MAP and in the two higher dose groups these changes obtained statistical significance. MAP was increased in 15 of 18 subjects within 5 min after the start of infusion. These dose levels did not have an effect on MAP in healthy volunteers. This may be of importance since other vasopressor agents tend to have a greater activity in normals than in septic shock patients. This suggests that PHP may have advantages over other agents due to its ability to directly target nitric oxide, the causative agent in shock. Further, it was noted that PHP had effects on MAP even in a population in which catecholamines, the current standard of care, were unable to stabilize blood pressure.
- The increases in MAP are mediated through changes in SVR. Human septic shock is characterized by a drop in SVR reflecting vasodilation induced by nitric oxide. As predicted by its mechanism of action of nitric oxide scavenging, PHP affects a change in MAP by reversing the NO-induced vasodilation and causing an increase in SVR. The SVR was increased in 17 of 17 patients by the end of the 30-min infusion.
- In the study population, vasopressor usage was reduced in 12 of the 18 patients.
- Urine output was increased in 10 of 14 patients in the period 1-h post-infusion compared to 1 h pre-infusion. Since low urine output in this population is in part related to low renal perfusion pressures, this result suggests that PHP can have a positive effect on restoration of normal renal perfusion.

- One of the basic concepts in the use of PHP for the treatment of shock is that nitric oxide overproduction is the result of a proinflammatory response that is independent of etiology. This is critical in enabling the selection of an appropriate patient population both for clinical trials as well as in its ultimate clinical use. In the group of 18 subjects, there were nine that had no positive cultures identified. Of the nine where positive cultures were detected, the isolates included a variety of gram-negative bacteria, gram-positive bacteria, and yeast. The sources of isolates included blood, urine, peritoneal fluid, and tracheal fluid.

These results support the concept that NO-induced shock is independent of etiology and that it will be possible to select a targeted patient population with a high probability of therapeutic activity.

Phase 2b

The Phase 2b [unpublished data] was an open-labeled ascending-dose study designed to assess the safety and tolerability of *PHP* in the presence of vasopressor infusion. A total of 23 patients were enrolled in this study and 22 were dosed. Patients were administered *PHP* as a continuous infusion at doses of:

- Cohort 1: 160 mg/kg (10–20 mg/kg/h, N = 4),
- Cohort 2: 320 mg/kg (10–40 mg/kg/h, N = 5),
- Cohort 3: 640 mg/kg (10–80 mg/kg/h, N = 6),
- Cohort 4: 2560 mg/kg (10–80 mg/kg/h, N = 7).

The protocol for this study required the participant to be volume-resuscitated and maintained on vasopressor therapy at the time of *PHP* infusion. The type(s) and number(s) of vasopressor(s) administered to the patient were not specified by the protocol, but by the conventional treatment at each site. The following four vasopressor/inotrope agents were administered to patients in the study: dopamine, norepinephrine, epinephrine, and phenylephrine. (Note: patients could be receiving more than one agent). The amount of each vasopressor administered was monitored for up to 168 h (7 days) post initiation of *PHP* infusion. Dobutamine and ≤5 μg/kg/min dopamine were not considered vasopressors for this study.

During the infusion of *PHP,* 21 of 25 vasopressor doses were reduced (4, 1, 12 and 4 for dopamine, epinephrine, norepinephrine and phenylephrine, respectively). Of the 21 vasopressors that were reduced, 9 were weaned (reduced to zero) (2, 5, and 2 for dopamine, norepinephrine and phenylephrine, respectively). Six patients were weaned from all standard vasopressors during *PHP* infusion.

An anticipated effect of *PHP* is reduction in the amount of vasopressor needed to maintain acceptable arterial blood pressure for patients with shock secondary to sepsis or presumed sepsis. At the start of *PHP* infusion, there were a total of 25 vasopressor doses being administered to the 22 patients. There were 4 patients receiving dopamine, 1 patient receiving epinephrine, 15 patients receiving norepinephrine, and 5 patients receiving phenylephrine.

Examined as a whole, the use of vasopressors decreased during the *PHP* infusion and for at least 24 h after the completion of the infusion. Cohort analysis revealed that all *PHP* dosage groups demonstrated reductions in vasopressor use during the 12 h window following introduction of *PHP*.
The effect of *PHP* on vasopressor usage was observed both during its infusion (from 8 to 53.5 h) and at least 24 h following the completion/stopping of the infusion.

The mean MAP for all patients at baseline was 69.3 ± 8.3 mmHg and means for the cohorts ranged between 64.5 and 75.3 mmHg. The median was 67.5 mmHg, the range was 55–90 mmHg, and 13 patients had baseline values less than 70 mmHg, the therapeutic target. The normal range for MAP is 70–105 mmHg. During the PHP infusion there were positive trends in most cohorts and statistically significant increases in MAP for all groups combined at 0.5, 1, 2, and 8 h after the start of PHP infusion despite the concomitant decrease in catecholamine dose. See Table 38.3 for summary of study.

Phase 3a

Following completion of the Phase 2b study the Data Safety Monitoring Board recommended that the development proceed to Phase 3. The FDA agreed and a Phase 3 protocol was accepted. It was designed as a phase 3 multicenter, randomized (1:1), placebo-controlled study but was terminated early due to poor enrollment. There was a high frequency of screen failures due to the inclusion criteria requiring a pulmonary artery catheter. The study was evaluated as a phase 2 study.

Table 38.3 Hemoximer summary of phase 2b study

PHP infusion permitted a decrease in vasopressor utilization while maintaining or increasing MAP
PHP infusion increased SVRI and decreased HR
PHP infusion was relatively safe and well-tolerated in shock patients
Resolution of shock following *PHP* infusion may be useful as an efficacy endpoint in future studies
SIRS and shock criteria select a patient population with elevated plasma nitrite + nitrate levels
Vasopressor activity of *PHP* is independent of etiology of shock or infectious agent
Vasopressor activity of *PHP* is consistent with a NO scavenging mechanism

The study was conducted at fifteen intensive care units in North America [65]. Sixty-two patients with distributive shock, >2 systemic inflammatory response syndrome criteria, and persistent catecholamine dependence despite adequate fluid resuscitation (pulmonary capillary wedge [65] pressure >12) were enrolled.

Randomized patients received either PHP at 0.25 mL/kg/h (20 mgHb/kg/h), or an equal volume of placebo, infused for up to 100 h, in addition to conventional vasopressor therapy. Treatment could not be blinded due to the color of PHP. Vasopressors and ventilatory support were weaned by protocol-defined procedures.

Enrollment of an appropriate population is a precondition for success in clinical trials. PHP is a NO scavenger that is designed to treat disorders where NO is present in excess. It is well documented in the literature that NO, a potent vasodilator, is the causative agent of distributive shock. Excess NO production is determined by measuring plasma nitrite plus nitrate (NOx) levels, the end products and biomarker of NO metabolism. If the target population was correctly selected, then they would have abnormally high levels of the NO biomarker. Normal levels of NOx are ~20 uM. The average NOx levels in the 62 patients enrolled was 120.3 uM in the PHP group and 110.6 uM in the placebo. This clearly demonstrates that the target population was selected with high precision.

The second goal of the study was to demonstrate the activity of PHP at the dose used in the study. Since it has been demonstrated that the enrolled patients have high NOx levels, PHP should reverse the vasodilation caused by the excess NO. This would mean that PHP could replace the catecholamines that patients were receiving. This can be measured in several ways. Since all the patients are receiving catecholamines at time 0, the time to first withdrawal of catecholamines should be shorter in the PHP group. Catecholamines were withdrawn from the PHP group an average of 12.55 h earlier than from the control (P = 0.07).

Another way to assess PHP vasoactivity is to determine the time alive and free of catecholamines. The PHP group was alive and free of catecholamines for an average of 30.2 h more than the placebo group. From day 6 to 16 the PHP group diverges from the placebo group. This shows that catecholamines are being withdrawn more rapidly in the PHP group. This data convincingly demonstrates that PHP is active as a vasopressor at this dose and can replace catecholamines, the standard of care for distributive shock.

The primary endpoints were tiered and look at both mortality and morbidity. Tier 1 is superiority of mortality; Tier 2 is noninferiority of mortality and superiority in cardiovascular and pulmonary function; Tier 3 is noninferiority in other organ function. Superiority of PHP in either Tier 1 or Tier 2 would demonstrate efficacy.

28-Day all-cause mortality was 57.6% for PHP and 58.6% for placebo. The unadjusted relative risk was 0.90 while the adjusted risk was 0.79. The lower adjusted risk was due to the covariates, in particular the APACHE II scores. This score showed that the PHP group was more severely ill at time 0 and had a higher predicted mortality that the placebo group. The SOFA scores also show the increased severity of illness in the PHP group at time 0.

It is also important to note that there was an early trend towards improved mortality. At day 7 there was a 15% difference between the PHP group and placebo (P = 0.27). A treatment for shock would be expected to work early in the course of illness, suggesting that PHP is working as expected based on its mechanism of action.

Tier 2 morbidity was measured by days alive and free of cardiovascular dysfunction and/or mechanical ventilation. A day free of cardiovascular dysfunction was a full 24-h period when a patient received no vasopressors and did not have a systolic blood pressure below 90 mmHg. The worst score was zero and the highest score is 56 days. If a patient died in the 28-day study period, they automatically received the worst score of zero. Since deaths receive the worst score of zero and the mortality in the PHP and placebo group were similar, the outcomes for the survivors drives the results. The PHP survivors group was free of cardiovascular dysfunction for 1.8 days more than the placebo group and free of mechanical ventilation for seven more days. The combined days alive and free of shock and vent for survivors was 8.8 days (P = 0.21).

Tier 3 is a safety endpoint designed to show that PHP has no adverse effect on other organ function such as liver, kidney, CNS, or coagulation. This was measured as days alive and free from medical interventions for treatment of organ dysfunction. The PHP group was numerically superior showing that PHP did not adversely affect these organs or require additional therapeutic intervention.

There were also other measures that supported the positive trends in morbidity. The PHP survivors group was in the ICU for 13.6 days and the placebo group for 17.9 days (P = 0.209). This is supporting evidence that the survivors in the PHP group are getting better faster and are not just lingering longer in the ICU and remaining critically ill. Shorter time in the ICU is a beneficial outcome for patients as well as for the pharmacoeconomics of PHP treatment.

In addition to the reduced ICU time, there is also a positive trend in hospital discharge rates for survivors in the PHP group. Eight of 14 PHP survivors were discharged by day 28 compared to 5 of 12 for the placebo.

In summary, the Phase 3a trial demonstrated that the targeted population was selected with high precision and using the Phase 3 dosing regimen, PHP was vasoactive. Furthermore, there were positive trends in all tiers of the primary efficacy, secondary, and safety endpoints.

Phase 3b

A redesigned multicenter, randomized, placebo-controlled, open-label phase 3 study [66] was conducted to compare the effectiveness and safety of PHP vs. placebo in patients with vasopressor-dependent distributive shock.

The study was conducted at sixty-one participating ICUs in six European countries (Austria, Belgium, Germany, the Netherlands, Spain, and United Kingdom).

Patients with distributive shock, defined as the presence of at least two systemic inflammatory response syndrome criteria, persisting norepinephrine dependence and evidence of organ dysfunction/hypoperfusion despite adequate fluid resuscitation were randomized to receive 0.25 mL/kg/h PHP (20 mg Hb/kg/h) or an equal volume of placebo, infused until resolution of shock or for up to 150 h, in addition to conventional vasopressor therapy.

The study was stopped for futility after interim analysis showed higher mortality in the PHP group and an increased prevalence of adverse events. At this time, 377 patients had been randomized to PHP (n = 183) or placebo (n = 194). Demographics and baseline measures were similar between groups. Twenty-eight-day mortality rate was 44.3% in the PHP group versus 37.6% in the placebo group (OR, 1.29; 95% CI, 0.85–1.95; p = 0.227). In patients with higher organ dysfunction scores (Sepsis-related Organ Failure Assessment > 13), mortality rates were significantly higher in the PHP group when compared with those in placebo-treated patients (60.9% vs 39.2%; p = 0.014). Survivors who received PHP had a longer vasopressor-free time (21.3 vs 19.7 d; p = 0.035). PHP decreased the need for vasopressors but was associated with a trend to increased mortality.

While the Phase 3 trial failed for futility with trends towards increased mortality, PHP performed exactly as designed and predicted. The hypothesis being tested was that PHP would scavenge NO and reduce mortality in a septic shock population with high NO.

The major weakness of the study was that there was no point of care mechanism to test for NO during the trial. A surrogate of high catecholamine was used, assuming the high catecholamine requirements correlated with high NO. This was supported by earlier observational/epidemiological studies. However, that assumption proved to be false and there were many subjects enrolled that had low NO.

In a subgroup analysis, the group with nitrate/nitrite above the median (the end-product of NO metabolism) had a non-significant trend towards improved mortality outcome. The group low nitrate/nitrite below the median had a non-significant trend towards worsened mortality outcomes. This result is consistent with the principal hypothesis that high NO levels are toxic and its corollary that further reduction of existing low levels of NO would be detrimental. This is a major lesson of the study and of significance for patient selection in any clinical trial of a hemoglobin-based therapeutic.

Conclusions

Two major challenges for the HBOC field have been the short half-life of HBOCs and the adverse effects associated with their use. The major adverse effect observed in most cases was increased blood pressure associated with vasoconstriction. In the late 1980s, following the discovery of NO as EDRF, the field began to make the connection between hemoglobin and NO. Apex Bioscience, originally founded to engineer hemoglobin in yeast, attempted to address the hemoglobin-NO interaction through protein engineering. The company ultimately adopted the viewpoint that hemoglobin is a multifunctional protein that carries oxygen, carbon dioxide, carbon monoxide and NO. In addition, hemoglobin possesses antioxidant properties to destroy NO, peroxynitrite and other reactive oxygen and nitrogen species.

Following extensive animal pharmacology studies and initial human clinical studies, PHP failed in its phase 3 trial. This was, at least in part, due to the inability to recruit subjects with elevated NO levels. Another factor was likely related to changes in standard of care including the common use of corticosteroids, an anti-inflammatory that would affect NO production. A third factor was country variation in standard of care and patient selection. For example, the placebo to PHP mortality was 27–44% in one country compared with 53–36% in another country. The study did demonstrate that PHP was an effective NO scavenger. But of greater importance it demonstrated that scavenging NO in patients with low NO levels could be detrimental and worsen outcomes.

This is an important lesson not just for the use of PHP as an NO scavenger but also for the use of HBOCs in general. The two phases of hemorrhagic shock, compensated vs. decompensated, have different patterns of vasoactive response. The compensated phase is a high catecholamine, low NO, vasocontrictive state. This is not an appropriate setting to give an NO scavenger. The decompensated phase is the reverse. It is a proinflammatory state [67] where NO is produced. Vasodilation causes blood pressure to decrease, thereby worsening the microcirculation and organ perfusion. This is potentially an optimal condition for administering an HBOC. It will deliver volume, oxygen, and reduce NO. Therefore, careful consideration of patient selection based on physiological and biochemical states and its relation to the multiple functions of hemoglobin is critical.

Key Points

- Hemoglobins are multifunctional proteins for gas/liquid transport but also possess antioxidant functions
- PHP is an effective NO scavenger in vivo
- Scavenging of NO by hemoglobins may be beneficial in states with excess NO
- Alternatively, NO scavenging in low NO states may be detrimental
- Clinical designs should take into account the multifunctionality of hemoglobin as well as the physiological state of subjects with respect to NO levels.

References

1. Iwashita Y. Hemoglobin conjugated with polyoxyethylene. In: Tsuchida E, editor. Artificial red cells. New York: Wiley; 1995. p. 151–76.
2. Szabo C. Alterations in nitric oxide production in various forms of circulatory shock. New Horiz. 1995;3:2–32.
3. Kilbourn RG, Griffith OW. Overproduction of nitric oxide in cytokine-mediated and septic shock. J Natl Cancer Inst. 1992;84:827–31.
4. Kilbourn RG, Jubran A, Gross SS, Griffith OW, Levi R, et al. Reversal of endotoxin-mediated shock by NG-methyl-L-arginine, an inhibitor of nitric oxide synthesis. Biochem Biophys Res Commun. 1990;172:1132–8.
5. Yokokawa K, Mankus R, Saklayen MG, Kohno M, Yasunari K, et al. Increased nitric oxide production in patients with hypotension during hemodialysis. Ann Intern Med. 1995;123:35–7.
6. Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hattler BG, et al. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. Science. 1992;257:387–9.
7. Parillo JE. Pathogenetic mechanisms of septic shock. N Engl J Med. 1993;328:1471–7.
8. Brady AJB, Poole-Wilson PA, Harding SE, Warren JB. Nitric oxide production within cardiac myocytes reduces their contractility in endotoxemia. Am J Phys. 1992;263:H1963–6.
9. Hibbs JB Jr, Vavrin Z, Taintor RR. L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J Immunol. 1987;138:550–65.
10. Mulligan MS, Hevel JM, Marletta MA, Ward PA. Tissue injury caused by deposition of immune complexes is L-arginine dependent. Proc Natl Acad Sci U S A. 1991;88:6338–42.
11. Van Dervort AL, Yan L, Madara PJ, Cobb JP, Wesley RA, et al. Nitric oxide regulates endotoxin-induced TNF-α production by human neutrophils. J Immunol. 1994;152:4102–8.
12. Wang Q, Jacobs J, DeLeo J, Kruszyna H, Kruszyna R, et al. Nitric oxide hemoglobin in mice and rats in endotoxic shock. Life Sci. 1991;49:PL55–60.
13. Kosaka H, Watanabe M, Yoshihara H, Harada N, Shiga T. Detection of nitric oxide production in lipopolysaccharide-treated rats by ESR using carbon monoxide hemoglobin. Biochem Biophys Res Commun. 1992;184:1119–24.
14. Westenberger U, Thanner S, Ruf HH, Gersonde K, Sutter G, et al. Formation of free radicals and nitric oxide derivative of hemoglobin in rats during shock syndrome. Free Rad Res Commun. 1990;11:167–78.
15. Gómez-Jiménez J, Salgado A, Mourelle M, Martin MC, Segura RM, et al. L-arginine: nitric oxide pathway in endotoxemia and human septic shock. Crit Care Med. 1995;23:253–8.
16. Ochoa JB, Udekwu AO, Billiar TR, Curran RD, Cerra FB, et al. Nitrogen oxide levels in patients after trauma and during sepsis. Ann Surg. 1991;214:621–6.
17. Cassoly R, Gibson QH. Conformation, co-operativity and ligand binding in human hemoglobin. J Mol Biol. 1975;91:301–13.
18. Moore EG, Gibson QH. Cooperativity in the dissociation of nitric oxide from hemoglobin. J Biol Chem. 1976;9:2788–94.
19. Doyle MP, Hoekstra JW. Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. J Inorg Biochem. 1981;14:351–8.
20. Gibson QH, Roughton FJW. The kinetics and equilibria of the reactions of nitric oxide with sheep hemoglobin. J Physiol. 1957;136:507–26.
21. Sharma VS, Ranney HM. The dissociation of NO from nitrosylhemoglobin. J Biol Chem. 1978;253:6467–72.
22. Sharma V, Traylor TG, Gardiner R, Mizukami H. Reaction of nitric oxide with heme proteins and model compounds of hemoglobin. Biochemistry. 1987;26:3837–43.
23. Addison AW, Stephanos JJ. Nitrosyliron(III) hemoglobin: autoreduction and spectroscopy. Biochemist. 1986;25:4104–13.
24. Alayash AI, Fratantoni JC, Bonaventura C, Bonaventura J, Cashon RE. Nitric oxide binding to human ferrihemoglobins cross-linked between either α or β subunits. Arch Biochem Biophys. 1993;303:332–8.
25. Alayash AI, Ryan AB, Fratantoni JC, Cashon RE. Redox reactivity of modified hemoglobins with hydrogen peroxide and nitric oxide: toxicological implications. Artif Cells Blood Substit Immobil Biotechnol. 1994;22:373–86.
26. Lancaster JR Jr. Simulation of the diffusion and reaction of endogenously produced nitric oxide. Proc Natl Acad Sci U S A. 1994;91:8137–41.
27. Winslow RM. Hemoglobin-based red cell substitutes. Baltimore: Johns Hopkins; 1992. p. 182–4.
28. Nakai K, Matsuda N, Amano M, Ohta T, Tokuyama S, et al. Acellular and cellular hemoglobin solutions as a vasoconstrictive factor. Artif Cells Blood Substit Immobil Biotechnol. 1994;22:559–64.
29. Rioux F, Petitclerc E, Audet R, Drapeau G, Fielding RM, et al. Recombinant human hemoglobin inhibits both constitutive and cytokine-induced nitric oxide-mediated relaxation of rabbit isolated aortic rings. J Cardiovasc Pharmacol. 1994;24:229–37.
30. Kida Y, Maeda M, Iwata S, Iwashita Y, Goto K, et al. Effects of pyridoxalalated hemoglobin polyoxyethylene conjugate and other hemoglobin related substances on arterial blood pressure in anesthetized and conscious rats. Artif Organs. 1995;19:117–28.
31. Agishi T, Funakoshi Y, Honda H, Yamagata K, Kobayashi M, Takahashi M. (Pyridoxalated hemoglobin)-(polyoxyethylene) conjugate solution as blood substitute for normothermic whole body rinse-out. Biomater Artif Cells Artif Organ. 1988;16:261–70.
32. Ajisaka K, Iwashita Y. Modification of human hemoglobin with polyethylene glycol: a new candidate for blood substitute. Biochem Biophys Res Commun. 1980;97:1076–81.
33. Iwasaki K, Iwashita Y. Preparation and evaluation of hemoglobin-polyethylene glycol conjugate (pyridoxalated polyethylene glycol hemoglobin) as an oxygen-carrying resuscitation fluid. Artif Organs. 1986;10:411–6.
34. Iwasaki K, Iwashita Y, Ikeda K, Uematsu T. Efficacy and safety of hemoglobin-polyethylene glycol conjugate (pyridoxalated polyethylene glycol hemoglobin) as an oxygen-carrying resuscitation fluid. Artif Organs. 1986;10:470–4.
35. Iwashita Y. Pyridoxalated hemoglobin-polyoxyethylene conjugate (PHP) as an oxygen carrier. Artif Organs Today. 1991;1:89–114.
36. Iwashita Y. Relationship between chemical properties and biological properties of pyridoxalated hemoglobin-polyoxyethylene. Biomater Artif Cells Immobilization Biotechnol. 1992;20:299–307.
37. Liu H, Agishi T, Suga H, Hayasaka Y, Teraoka S, Ota K. The effect of total blood exchange with PHP solution on cardiac xenotransplantation. Artif Organs. 1995;19:324–7.

38. Matsushita M, Yabuki A, Chen JF, Takahashi T, Harasaki H, Malchesky PS, Iwashita Y, Nosé Y. Renal effects of a pyridoxalated-hemoglobin-polyoxyethylene conjugate solution as a blood substitute in exchange transfusions. ASAIO Trans. 1988;34:280–3.
39. Matsushita M, Yabuki A, Malchesky PS, Harasaki H, Nosé Y. *In vivo* evaluation of a pyridoxalated-hemoglobin-polyoxyethylene conjugate. Biomater Artif Cells Artif Organs. 1988;16:247–60.
40. Privalle C, Talarico T, Keng T, DeAngelo J. Pyridoxalated hemoglobin polyoxyethylene: a nitric oxide scavenger with antioxidant activity for the treatment of nitric oxide-induced shock. Free Radic Biol Med. 2000;28:1507–17.
41. Talarico T, Guise K, Stacey S. Chemical characterization of pyridoxalated hemoglobin polyoxyethylene conjugate. Biochim Biophys Acta. 2000;1476:53–65.
42. DeAngelo J. Nitric oxide scavengers in the treatment of shock associated with systemic inflammatory response syndrome. Expert Opin Pharmacother. 1999;1:19–29.
43. Talarico T, Swank A, Privalle C. Autoxidation of pyridoxalated hemoglobin polyoxyethylene conjugate. Biochem Biophys Res Commun. 1998;250:354–8.
44. Miles P, Langley K, Stacey C, Talarico T. Detection of residual polyethylene glycol derivatives in pyridoxalated-hemoglobin-polyoxyethylene conjugate. Artif Cells Blood Substit Immobil Biotechnol. 1997;25(3):315–26.
45. Privalle C, Keng T, DeAngelo J, Talarico T. Pyridoxalated hemoglobin polyoxyethylene (PHP): a modified hemoglobin-based nitric oxide scavenger with antioxidant activity for the treatment of shock associated with systemic inflammatory response syndrome (SIRS). Nitric Oxide. 2002;6:450.
46. Privalle C, Keng T, DeAngelo J, Allen J, Talarico T. Antioxidant properties of pyridoxalated hemoglobin polyoxyethylene (PHP): a modified hemoglobin-based nitric oxide scavenger for the treatment of NO-induced shock. Free Radic Biol Med. 2000;29(Suppl 1):S74.
47. Privalle C, Keng T, DeAngelo J, Talarico T. Pyridoxalated hemoglobin polyoxyethylene (PHP): a nitric oxide scavenger with SOD-catalase activity for the treatment of nitric-oxide induced shock. Nitric Oxide. 2000;4:206.
48. Privalle C, Keng T, Talarico T. Pyridoxalated hemoglobin polyoxyethylene (PHP) – a modified hemoglobin therapeutic with SOD-catalase activity. Free Radic Biol Med. 1999;27(Suppl 1):S85.
49. Privalle C, Keng T, DeAngelo J, Talarico T. Pyridoxalated hemoglobin polyoxyethylene (PHP): a nitric oxide scavenger containing SOD and catalase which reduces hemoprotein-mediated redox reactivity following oxidant challenge. Acta Physiol Scand. 1999;167(Suppl 645):200.
50. Privalle C, Keng T, DeAngelo J, Talarico T. Pyridoxalated hemoglobin polyoxyethylene (PHP): nitric oxide scavenging and antioxidant properties. Free Radic Biol Med. 1998;25(Suppl 1):S65.
51. Privalle C, Keng T, DeAngelo J, Talarico T. Pyridoxalated hemoglobin polyoxyethylene (PHP): a hemoglobin-based nitric oxide scavenger with antioxidant properties. Nitric Oxide. 1998;2:79.
52. Privalle C, Talarico T, DeAngelo J, Keng T. Interactions of nitric oxide and peroxynitrite with hemoglobin and PHP. Jpn J Pharmacol. 1997;75(Suppl 1):109.
53. Privalle C, Talarico T, DeAngelo J, Keng T. Interactions of nitric oxide and peroxynitrite with hemoglobin and PHP. In: Moncada S, Toda N, Maeda H, Higgs EA, editors. The biology of nitric oxide, Part 6. London: Portland Press; 1998. p. 302.
54. Privalle C, Keng T, DeAngelo J, Talarico T. Pyridoxalated hemoglobin polyoxyethylene conjugate (PHP): a nitric oxide scavenger containing SOD and catalase which reduces hemoprotein-mediated redox reactivity following oxidant challenge. In: Moncada S, Gustafsson L, Wiklund P, Higgs EA, editors. The biology of nitric oxide, Part 7. London: Portland Press; 2000. p. 146.
55. Bone H, Schenarts P, Booke M, McGuire R, Harper D, Traber L, Traber D. Pyridoxalated hemoglobin polyoxyethylene conjugate normalizes the hyperdynamic circulation in septic sheep. Crit Care Med. 1997;25:1010–8.
56. Kilbourn R, DeAngelo J, Bonaventura J. Clinical effects of cell-free hemoglobin, a scavenger of nitric oxide, in septic shock. In: Vincent JL, editor. The yearbook of intensive care and emergency medicine 1997. Brussels: Springer; 1997. p. 230–9.
57. Fischer S, Bone H, Traber D. Effects of hemoglobin in sepsis. In: Vincent JL, editor. The yearbook of intensive care and emergency medicine 1997. Brussels: Springer; 1997. p. 424–41.
58. Fischer S, Bone H, Powell W, McGuire R, Traber L, Traber D. Pyridoxalated hemoglobin polyoxyethylene conjugate (PHP) does not restore hypoxic pulmonary vasoconstriction in ovine sepsis. Crit Care Med. 1997;25:1551–9.
59. Bone H, Waurick R, Van Aken H, Booke M, Prien T, Meyer J. Comparison of the haemodynamic effects of nitric oxide synthase inhibition and nitric oxide scavenging in endotoxaemic sheep. Intensive Care Med. 1998;24:48–54.
60. Bone H, Schenarts P, Fischer S, McGuire R, Traber L, Traber D. Pyridoxalated hemoglobin polyoxyethylene conjugate reverses hyperdynamic circulation in septic sheep. J Appl Physiol. 1998;84:1991–8.
61. Bone H, Fischer S, Schenarts P, McGuire R, Traber L, Traber D. Continuous infusion of pyridoxalated hemoglobin polyoxyethylene conjugate in hyperdynamic sheep. Shock. 1998;10:69–76.
62. Fischer S, Traber D. L-Arginine and endothelin receptor antagonist bosentan counteract hemodynamic effects of modified hemoglobin. Shock. 1999;11:283–90.
63. Fischer S, Powell P, McGuire R, Traber L, Traber D. Submitted. Pyridoxalated hemoglobin polyoxyethylene conjugate (PHP) decreases catecholamine requirements in ovine sepsis. Crit Care Med.
64. Bone H, Waurick R, Van Aken H, Jahn U, Booke M, Meyer J. The hemodynamic effects of cell-free hemoglobin during general and epidural anesthesia. Anesth Analg. 1999;89:1–6.
65. Kinasewitz GT, Privalle CT, Imm A, Steingrub JS, Malcynski JT, Balk RA, DeAngelo J. Multicenter, randomized, placebo-controlled study of the nitric oxide scavenger pyridoxalated hemoglobin polyoxyethylene in distributive shock. Crit Care Med. 2008;36(7):1999–2007. https://doi.org/10.1097/ccm.0b013e31817bfe84. PMID: 18552688.
66. Vincent JL, Privalle CT, Singer M, Lorente JA, Boehm E, Meier-Hellmann A, Darius H, Ferrer R, Sirvent JM, Marx G, DeAngelo J. Multicenter, randomized, placebo-controlled phase III study of pyridoxalated hemoglobin polyoxyethylene in distributive shock (PHOENIX). Crit Care Med. 2015;43(1):57–64. https://doi.org/10.1097/CCM.0000000000000554. PMID: 25083980.
67. Hierholzer C, Harbrecht B, Menezes JM, Kane J, MacMicking J, Nathan CF, Peitzman AB, Billiar TR, Tweardy DJ. Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. J Exp Med. 1998;187(6):917–28. https://doi.org/10.1084/jem.187.6.917. PMID: 9500794; PMCID: PMC2212185.

Part V

Specific Indications, Regulatory Issues and Future Directions

Hemoglobin-Based Oxygen Carrier Solutions for Organ and Tissue Preservation and Transplantation

39

Paulo A. Fontes, William Rick Light, Arjan van der Plaats, Elyse M. Cornett, and Alan D. Kaye

Introduction

Machine Perfusion for Organ and Tissues Preservation

Organ perfusion was first attempted in mid-nineteenth century by Loebel, who conducted preliminary perfusion studies of isolated organs in 1849. Langendorf developed a simple and non-pulsatile organ perfusion apparatus where a perfusate reservoir and a siphon tube were attached to the organ in 1895. The organ was perfused by gravity in an open system without recirculation of the perfusate [1]. Knowlton and Starling introduced the first heart-lung preparation in 1912, by combining active perfusion with blood oxygenation [2]. Bainbridge and Evans reported the first successful kidney perfusion experiments in 1914. They achieved sustainable blood flow *ex vivo* while keeping the temperature and vasculature pressures within physiological ranges. This normothermic kidney perfusion yielded normal arterial blood gases values while allowing the kidney to produce urine [3].

Carrel joined Rosenberg in 1930 to develop a new automated perfusion system composed of a combined metal and glass pump. Organ perfusion experiments became rather routine during the second and third decades of twentieth century. Charles Lindbergh devised a new all-glass apparatus at the Rockefeller institute in 1935, becoming the first one to provide flow under pulsatile pressures and within sterile conditions [4]. In the mid-1960s the Naval Medical Research Institute, MD, USA became interested in ex vivo organ perfusion. Lindbergh was brought in to assist the research institute, where he successfully reproduced and validated his previous experiments.

Kidney transplantation was clinically attempted in the early 1960s despite the lack of progress in organ preservation. Early attempts to induce total body hypothermia in living kidney donors were performed with the technology initially developed for open heart procedures [5]. Hypothermia was further utilized by Lillehei to extend organ preservation by immersing small bowel loops in iced saline before doing autotransplants [6]. Hypothermia was readily accepted as beneficial for organ preservation in the 1960s, despite the lack of technological developments in solutions and machine perfusion (MP) devices. Subsequent preclinical experiments in dogs showed the beneficial effect of hypothermia by infusing cold solutions through the portal vein in liver allografts prior to transplantation.

The same principle was applied in clinical kidney transplantation, where cold lactated Ringer's or low-molecular-weight dextran solutions were infused through the renal artery in kidney allografts immediately after the initial procurement. Continuous hypothermic perfusion of cadaveric livers and kidneys allografts became routine long before brain death was accepted as a clinical condition for organ donation. The first human liver transplant in 1963 was con-

P. A. Fontes (✉)
LyGenesis Inc., Pittsburgh, PA, USA
e-mail: fontesp@lygenesis.com

W. Rick Light
VirTech Bio, Natick, MA, USA
e-mail: Rick.Light@virtechbio.com

A. van der Plaats
XVIVO Perfusion, Groningen, Netherlands
e-mail: arjan.vanderplaats@xvivogroup.com

E. M. Cornett
Department of Anesthesiology, LSU Health Shreveport, Shreveport, LA, USA
e-mail: elyse.bradley@lsuhs.edu

A. D. Kaye
Department of Anesthesiology and Department of Pharmacology, Toxicology, and Neurosciences, LSU School of Medicine, Shreveport, LA, USA

Department of Anesthesiology and Pharmacology, LSU School of Medicine, New Orleans, LA, USA

Department of Anesthesiology and Pharmacology, Tulane School of Medicine, New Orleans, LA, USA
e-mail: alan.kaye@lsuhs.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_39

ducted after the cadaveric allograft was perfused *ex vivo* by a machine perfusion developed by Starzl and his Denver group. Ackerman and Barnard reintroduced the Carrel/Lindberg MP system by perfusing kidney allografts with continuous low flow using a perfusate primed with blood and oxygenated within a hyperbaric chamber [7]. *Ex vivo* oxygenation appeared to be a feasible technique and it was further utilized for the preservation of hepatic allografts [8].

Belzer discarded the use of hyperbaric chamber in combination with blood products and developed a new preservation solution for CS, showing remarkable results in a 72-hour kidney preservation model [9]. Their new solution was further optimized as the first blood-free perfusate for MP. Belzer's team introduced hypothermic machine perfusion (HMP) using cryoprecipitated plasma as the perfusate in a protocol involving 17-hour preservation prior to kidney transplantation [10]. They extended their studies with HMP and developed a hyperconcotic heterostarch-based solution, the University of Wisconsin machine perfusion solution (MPS), which became the gold standard MPS for several decades [11]. Pienaar achieved a remarkable 87.5% survival in a liver transplant dog model where they utilized HMP for 72 hours, using pulsatile perfusion through the portal vein with high pressures (16–18 mmHg) and switching the Na+/K+ ratio of their original MPS solution [12].

Subsequent developments in MP were further promoted by Dutkowski, who introduced the concept of end-ischemic hypothermic oxygenated perfusion (HOPE) for liver preservation where they reintroduced MP for a short period prior to organ implantation [13]. Their extensive studies in rodents and preclinical large animal models showed their ability to reduce endothelial shear stress while minimizing the release of reactive oxygen species (ROS) by providing a short period (1–3 hours) of oxygenation under hypothermic conditions. They developed a pressure-sealed chamber capable to deliver intermittent positive/negative pressure with an amplitude of 8 mbar, producing physiological flow profiles between 32–36 mL/minute. This fluctuating and oscillating pressure-controlled perfusion through the portal vein, was thought to progressively restore mitochondrial function and promote effective ATP reconstitution during a short but effective perfusion time [14, 15]. Normothermic (37 °C) MP was also reintroduced by Neuhaus [16] in 1993 and further optimized by Friend [17] in 2009. HMP (4 °C) was reintroduced again by Guarrerra in 2005, who further pioneered the clinical applications in MP in the USA by conducting a successful feasibility trial with 20 patients in 2009 [18]. A subsequent clinical trial was conducted by Friend in Oxford, UK, where a new NMP device utilizing human red blood cells (RBCs) as the OC solution [19].

Fontes et al. developed the first use of modified hemoglobin-based oxygen carrier (HBOC) solutions in combination with MP [20] in swine liver transplantation experiments in which the novel MP/HBOC system was compared with cold static preservation. Liver preservation has been performed within a wide temperature range while migrating from 4 °C (cold storage) to full MP at 37 °C (normothermic). Interesting developments in mitochondria function, ATP reconstitution and cell membrane integrity have paved the way to a new understanding of the true biological impact of prolonged ischemic insult following the initial liver procurement. This chapter summarizes recent developments in MP for liver and vascularized composite allografts (VCA) preservation. We highlight our experience in 21 °C (subnormothermic) MP, while emphasizing the biological implications in the metabolism and function when effective oxygenation is provided ex vivo with hemoglobin-based oxygen carrier (HBOC) solutions. We also share our recent developments in data analysis, where computational biology tools are used to integrate data from transcriptomics, inflammatory markers, and metabolomics.

Rationale for the Use of Machine Perfusion Devices

MP devices have emerged to provide sustained flow (continuous and/or pulsatile) into organ allografts to enhance/extend organ preservation while reducing ischemia reperfusion damage [21]. The following are the primary issues at stake in MP:

1. Constant circulation and improved flow through the macro- and microcirculations
2. Effective oxygenation to meet/repair the metabolic demands of the organ
3. Elimination of metabolic byproducts and poisons
4. Provide a chance to evaluate the function and viability of organs.
5. Improve clinical results by encouraging ex vivo organ rescue/amelioration prior to implantation and allowing for the use of donors who meet enlarged criteria (ECDs),
6. Extend preservation time without imposing further ischemia-reperfusion injuries (IRIs),
7. Promote the *ex vivo* administration of cytoprotective and immunomodulatory substances,
8. Improve transplant outcomes by decreasing the incidence of early graft dysfunction and PNF, which would impact directly on the length of hospital stay, subsequent posttransplant admissions and improved graft and patient survival rates. See Table 39.1.

Table 39.1 Risks & benefits of MP

Advantages	Disadvantages
Lower incidence of delayed graft function	Higher cost and enhanced logistics
Continuous monitoring of parameters	New requirements for organ manipulation ex-vivo
Decrease vasospasm when avoiding prolonged hypothermia	Endothelial injury is possible if flows are not properly controlled
Ability to provide metabolic support	New requirements for ex-vivo monitoring and the development of new biomarkers
Potential for pharmacologic manipulation	Possible equipment failure
Immunomodulation	Infection
Control of inflammation (DAMPs)	
Development of new biomarkers to monitor and predict organ function	Increase costs in organ preservation

Developing HBOCs for Specific Ex-Vivo Utilization in Organ and Tissue Preservation

Machine perfusion with an oxygen carrier has the potential to narrow the gap between organ supply and demand. Acellular Hemoglobin-Based Oxygen Carriers (HBOCs) have several acknowledged advantages as an *ex vivo* storage solution (versus blood) including ease of use (no blood typing), simplified storage logistics (room temperature stable for years), efficient oxygenation over a wide range of temperatures, and sterility. And unlike blood *in vivo* where coagulation is required, the technical and operational problems related blood coagulation *ex vivo* can be totally avoided by the use of HBOC in combination with MP.

W. Richard Light (Rick) and Paulo Fontes developed and patented (WO2014059316 A1, PCT/US2013/064607) a new Hemoglobin Based Oxygen Carrier (HBOC) solution that was successfully utilized as a new perfusate for a machine perfusion (MP) system with dual pressures and under subnormothermic conditions (21 °C) [20]. This new solution was based on the combination of bovine-based HBOC-201 with a heterostarch (HES) based colloid solution (Belzer MPS – BMPS, Preservation Solutions Inc., Elkhorn, WI) that added several new properties to the original HBOC-201 solution as a more effective promoter of organ preservation:

(a) Increased oncotic pressure by the addition of impermeants and colloids
(b) Addition of 2 new buffers (H_2PO_4 and Hepes)
(c) Alteration of the Na^+/K^+ ratio
(d) Addition of free radical scavengers (glutathione and allopurinol)
(e) Addition of insulin as a liver growth factor
(f) Addition of glucose as a nutrient
(g) Addition of a purine nucleobase (adenine) as a cofactor for cellular respiration
(h) Addition of a pentose (ribose) as an energy substrate
(i) Decrease (dilutional effect) of the original hemoglobin concentration from 13 g/dl to 3 g/dl.

Related to major regulatory issues with FDA regarding bovine-derived products, a new human-based HBOC was subsequently designed (OxyBridge™, VirTech Bio, Natick, MA) with following additional benefits:

1. the hemoglobin (Hb) is human-derived, decreasing major CMC regulatory concerns from previous IDE applications with the FDA.
2. the Hb polymer (OxyBridge) is larger than most other HBOCs (1600 kD versus 250 kD) to minimize extravasation,
3. unique solution properties (COP and viscosity) modeled after pRBCs,
4. cost-effective manufacturing able to be implemented at a Contract Manufacturing Organization (CMO)to maximize the HBOC clinical and commercial potential.

OxyBridge is formulated in a balanced salt solution similar to Ringer's lactate. The resulting purified hemoglobin solution does not contain any cells and has no issues regarding xeno antigens for subsequent use in human tissues [5]. OxyBridge is human derived, glutaraldehyde polymerized, with a higher molecular weight (MW ≈ 1600 kD, p50 = 36 mmHg) and is engineered for cost-effective production through scalable filtration technology to be Contract Manufacturing Organization 'friendly'.

OxyBridge has a total oxygen-carrying capacity like that of native, corpuscular hemoglobin, but the average OxyBridge molecule is less than 1/100,000,000th the volume of a red blood cell. Upon infusion, this stabilized hemoglobin distributes throughout the plasma. Thus, OxyBridge increases the oxygen content of the intravascular solution, 'plasma'. OxyBridge plasma is in continuous contact with blood artery walls, where oxygen is transported to tissues. Plasma circulates in all locations where blood normally circulates and can even circumvent partial blockages or pass-through constricted arteries that are too narrow to carry red blood cells normally. The result is that OxyBridge facilitates enhanced diffusive oxygen delivery while improving tissue oxygenation more efficiently than non-hemoglobin crystalloid and colloid preservation solutions. OxyBridge can bind the same amount of oxygen as the hemoglobin molecules in red blood cells, on a gram-for-gram basis, and release oxygen more readily than red blood cells.

OxyBridge is formulated at a concentration (11 g/dl) to facilitate mixing with standard organ storage solutions to

match the specific requirements of a particular organ. For the liver perfusion experiments, OxyBridge was combined with Belzer machine perfusion solution (BMPS, Preservations Solutions Inc., Elkhorn, WI). For other organs, additional mixing solutions can potentially be used. BMPS is a modification of the University of Wisconsin solution (UW), which has been the gold standard for organ preservation around the world since 1989 [11]. BMPS was originally developed in 1986 and it has been extensively used in machine perfusion applications for kidneys and livers. BMPS has been used as a modified solution (Vasosol) in more than 20 patients who received liver allografts preserved with an hypothermic machine perfusion system (HMP) [18].

Pharmacodynamics (PD) and Pharmacokinetic (PK) Experiments with OxyBridge

Additional PK/PD and validation experiments were conducted in our lab using a 12-hour liver perfusion model with MP. Three liver allografts were obtained from Landrace pigs (average weight = 60 kg) within standard operative techniques for organ recovery in clinical transplantation. The livers were perfused in our MP system for 12 hours at 21 °C using **OxyBridge** as the perfusate in combination with Belzer UW® MPS. The liver allografts received dual inflow, with pulsatile flow in the hepatic artery (map = 30 mmHg) and continuous flow in the portal vein (mvp = 4 mmHg). The pH of the perfusate was maintained by repeated measurement of blood gases and adjustments of the FiO2 and sweep gas rates through the oxygenators using common clinical practices. Pharmacokinetic (PK) characterizations were performed by verifying the half-life of the HBOC solution by sampling at pre-determined times during perfusion of the isolated livers. The hemoglobin content was also measured overtime. PD characterizations were performed by establishing O_2 dissociation (p50) curves. Additional measurements of oxidative phosphorylation via mitochondrial functional assays were also performed. Direct and indirect measures of oxygen delivery and oxygen consumption by the liver allografts were obtained. Subsequent measurements of the oncotic pressure, viscosity, osmolality, electrolyte composition, *c*THb, *F*O_2Hb, *F*HHb, *F*MetHb and MW distribution were further obtained.

Perfusate (OxyBridge) Gas Analysis

Each sample of the perfusate solution obtained for blood gas analysis was obtained in a 1 mL tuberculin syringe and capped until analysis to prevent gas exchange prior to analysis. Samples were loaded into the ABL Flex 800 (Radiometer, Copenhagen) blood gas analyzer. Sample pH and pCO_2 was temperature correct to the recorded OALD system temperature of 21 °C. pH (mmHg), pO_2 (mmHg), pCO_2 (mmHg), K^+ (mmol/L), Cl^- (mmol/L), Na^+ (mEq/L), Ca^{2+} (mmol/L), Lactate (mmol/L), and Glucose (mmol/L) were measured via electrode chemistry. Hb (g/dL), sO_2 (%), FO_2Hb (%), FCOHb (%), and FMetHb (%) were measured on the ABL 800 Flex via color oximetry.

Hemoximetry

Oxy-Hb association and dissociation curves for the OxyBridge solution obtained for hemoximetry were analyzed using the Hemox Analyzer (Model B, TCS Scientific Corp, New Hope, PA). All measurements for hemoximetry were performed at 37 °C. Briefly, each sample of the HBOC solution were warmed to 37 °C prior to sample preparation. Two hundred fifty microliters of the HBOC solution sample was added to a clean polystyrene cuvette containing 4 mL of Hemox Solution (TCS) and 20 μL of anti-foaming agent A (TCS). The contents of the cuvette were mixed and loaded into the sample analyzer, which consisted of a glass chamber with a magnetic stir bar in the bottom. A precise mix of nitrogen and oxygen was bubbled through the solution and measurements of the log-transformed optical density at 560 nm (measuring wavelength) and 570 nm (isosbestic wavelength) were made. The p50, which is the partial pressure of oxygen where 50% of the Hb molecules contain oxygen, was obtained using a built-in software algorithm for the Hemox Analyzer and recorded. The raw data from the oximetry was exported to a spreadsheet package (Microsoft Excel 2010, Microsoft Corporation, Seattle, WA) for graphical representation of the average saturation and partial pressure of oxygen over the time course of samples for each experiment.

Results: Perfusion Parameters Ex-Vivo

Machine perfusion was conducted with full oxygenation (FiO2 = 60%). Perfusate's oxygenation was kept in the 550–650 mmHg range in the arterial port, where an oxygen saturation of 90% was obtained. The venous port was kept in the 250–450 mmHg range, where an oxygen saturation of 70% was consistently maintained. The systolic arterial pressures (hepatic artery) were kept at 30 mmHg, and the venous pressures (portal vein) were kept at 4 mmHg. The perfusion flows were kept between 300–600 ml/min in the PV and 100–200 ml/min in the hepatic artery. There were no signs of vasoconstriction as seen in previous in-vivo experiments with the HBOC-201 when this component was utilized in much higher concentration (13 g/dL) and without the presence of colloids and additional scavengers. The liver allografts had clear signs of progressive vasodilatation as the

arterial flows (through the hepatic artery) showed a 150% increase over the course of the experiments. The perfusate temperature was kept steadily at 21 °C. The pH was kept within a slightly alkalotic range (7.50–7.58). No $NaHCO_3$ infusions were required to keep the pH within the physiological range. Liver allografts cleared lactate (dropping down to values around zero) and produced bile consistently throughout the duration of the experiments (12 hours). Oxygen saturation reached 100% with normal pO_2 values.

Mitochondrial Function

Fresh liver tissue was obtained during preservation and mitochondrial functional analyzed in a sealed oxygraph chamber fit with a Clark-type oxygen electrode (Instech Laboratories Inc., Plymouth Meeting, PA, USA) connected to a data recording device (DATAQ Systems) [1]. The respiratory control ratio (RCR), which is well accepted as the most useful general measure of function of isolated mitochondria was normal and stable throughout the entire duration of the experiments, revealing adequate oxidative phosphorylation by the tissues perfused with the HBOC. Basal ATP generation rate was quantified to determine whether energetics were sufficient throughout the experiment. Additionally, mitochondrial hydrogen peroxide production was measured as an indicator of ROS generation in the tissue (a natural response to ischemia/reperfusion) [22].

Cell-free hemoglobin, in either its oxygenated or deoxygenated state, can react almost instantaneously with NO ($k = \sim 107\ M{-}1 \cdot s{-}1$) resulting in hypertension when coupled with the relatively high concentrations of heme (at the mM level) arising from HBOC administration during resuscitative therapies through a significant inhibition of local NO signaling within the endothelium. In order to assess additional pathways that could trigger further hemoglobin oxidation leading into methemoglobinemia, nitrite-dependent oxidation was serially assessed every 3 hours during the 12-hour perfusion protocol. The major products of the nitrite/oxyHb reaction are nitrate, metHb, and H_2O_2 (ROS). Nitrate is a benign component but metHb and ROS can be further reduced by metHb reductase and dismutated by catalase, respectively. Nitrite reduction can take place from a direct reaction with the ferrous heme group of deoxygenated hemoglobin. Further reaction of nitrite with oxyhemoglobin (oxyHb) generates nitrate and methemoglobin (metHb).

Nitrite and Nitrate Measurements

Nitrite and nitrate concentrations were measured by reductive chemiluminescence as previously described [23]. Briefly, nitrite was measured by injecting samples into a purge vessel containing a solution of triiodide, connected in line to a Nitric Oxide Analyzer (Sievers, GE). Nitrate was measured by injecting identical samples into a vessel purged with helium containing vanadium chloride to reduce nitrate to NO. The NO was detected by the Nitric Oxide Analyzer.

Mitochondrial Respiration Measurements

Mitochondria were isolated from liver by differential centrifugation and resuspended in Respiration buffer (120 mM KCl, 25 mM sucrose, 10 mM HEPES, 1 mM EGTA, 1 mM KH_2PO_4, 5 mM $MgCl_2$). for oxygen consumption measurements utilizing a Clark electrode as previously described [14]. Briefly, mitochondria were suspended at 1 mg/ml and oxygen consumption monitored after the addition of succinate (10 mM) and subsequently ADP (25 mM). Respiratory control ratio was found by expressing State 3 (ADP dependent rate) over State 4 (succinate dependent rate) [24].

Reactive Oxygen Species (ROS) Measurements

Hydrogen peroxide (H_2O_2) generation was quantified in isolated mitochondria by measuring the rotenone sensitive oxidation of Amplex Red spectrophotometrically as previously described [25]. In the presence of nitrite, the nitrite reductase activity of deoxyhemoglobin creates a balance between NO formation and haem-based NO scavenging that dictates the degree to which NO can stimulate signaling and vasodilation.

Results

Four different hemoglobin (Hb) species are commonly recognized: oxyhemoglobin (oxy-Hb), deoxyhemoglobin (deoxy-Hb), and methemoglobin (met-Hb). During the 12-hour perfusion there was no change in total Hb concentration, which indicates the lack of precipitation and/or filtering during the machine perfusion procedure (Fig. 39.1).

The % of oxyhemoglobin was also sustained over the 12-hour period. Adequate oxygen delivery depends on the hemoglobin binding, transporting, and ultimately unloading O2 molecules. Each hemoglobin can bind zero oxygens, one, two, three, or four. This binding rate is directly related to oxygen content dissolved in perfusate. The higher the oxygen content in solution (PaO2), the higher the hemoglobin binding (SaO2). **OxyBridge** was able to maintain a high percentage of oxyhemoglobin over the 12-hour period, which means that it sustained a high degree of oxygen delivery overtime to the hepatic tissue (Fig. 39.2).

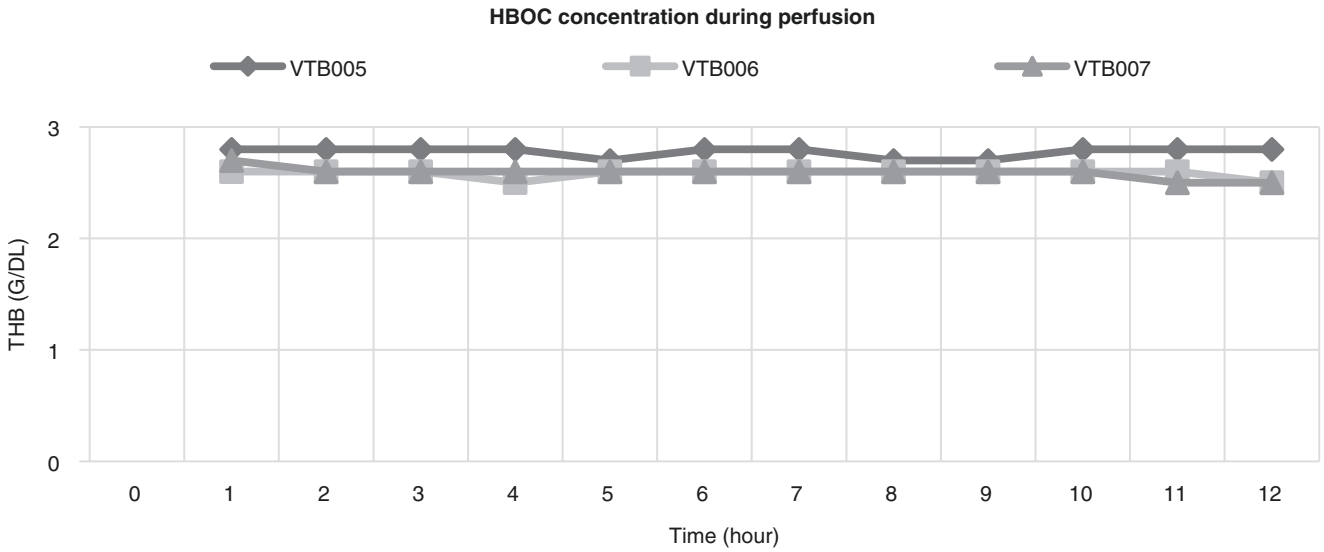


Fig. 39.1 Hb concentration (g/dL) during the 12-hour machine perfusion liver preservation protocol

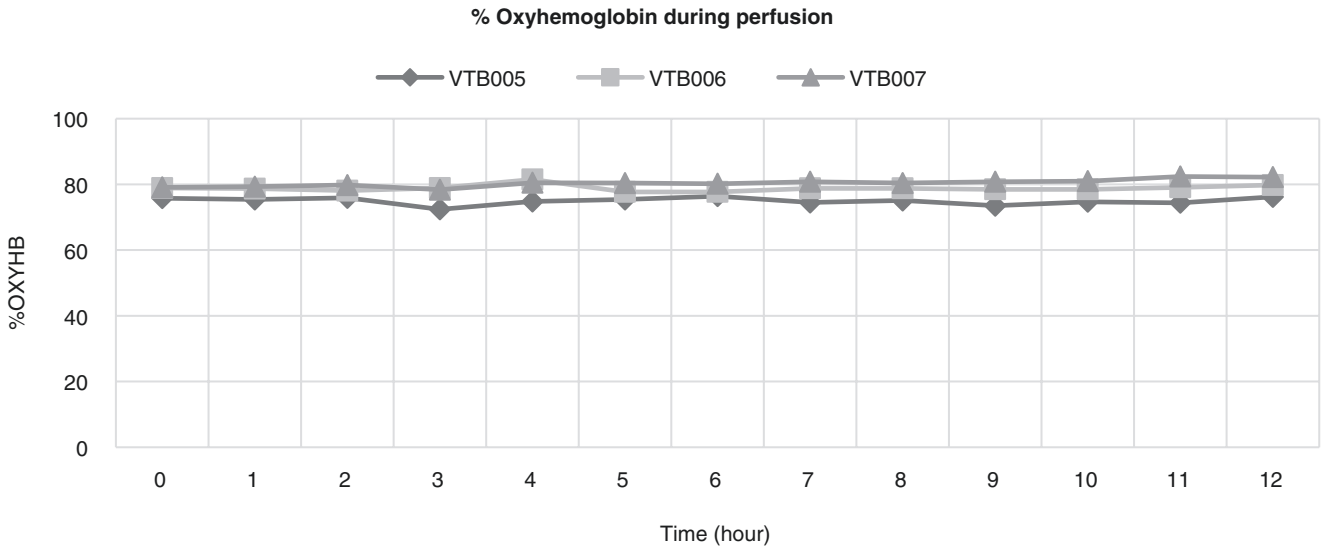


Fig. 39.2 Percent oxyhemoglobin during the 12-hour machine perfusion liver preservation protocol

Methemoglobin (MetHb) is a naturally occurring oxidized metabolite of hemoglobin that decreases its ability to bind oxygen. MetHb contains iron in the ferric state (Fe^{3+}) rather than the reduced ferrous form (Fe^{2+}) found in hemoglobin. % MetHb measures the inactive form of the protein and is used as a stability indicator. This undesirable oxidation can be exacerbated by time and packing issues. **OxyBridge** show levels of MetHb lower than 3 in the bag and lower than 15 in the perfusate during machine perfusion (See Fig. 39.3).

The pO2 was continuously monitored in the perfusate with a ABL Flex 800 (Radiometer, Copenhagen) blood gas analyser. The machine perfusion protocol was conducted under a FiO2 = 60% at 21 °C. In spite having a low Hb (3 g/dL) concentration in the perfusate, the pO2 were consistently high over the 12-hour period. MetHb is a naturally occurring oxidized metabolite of hemoglobin that does not bind oxygen and creates detrimental reactive oxygen species (ROS). To assess pathways that could trigger OxyBridge oxidation to metHb, nitrite-dependent oxidation was assessed every 3 hours during the 12-hour perfusion protocol. The major products of the nitrite/oxyHb reaction are nitrate, metHb, and H_2O_2 (ROS). Nitrite reduction can take place from a direct reaction with the ferrous heme group of deoxygenated hemoglobin. Further reaction of nitrite with oxyhemoglobin (oxyHb) generates nitrate and metHb. Compared to data pre-

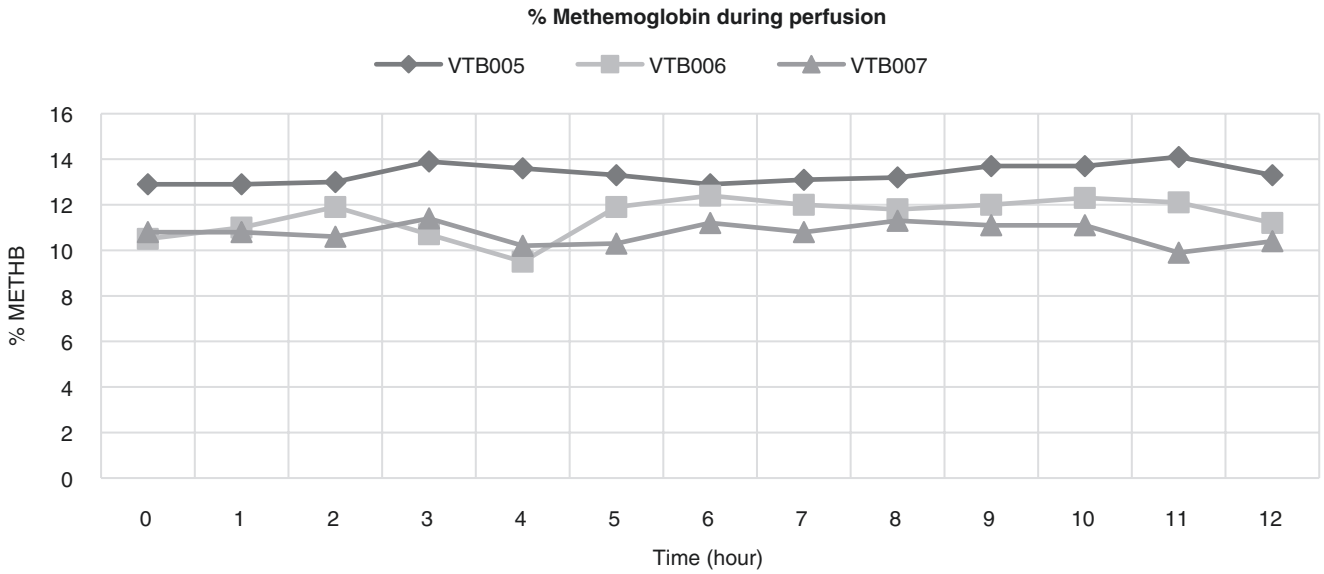


Fig. 39.3 Percent methemoglobin during the 12-hour machine perfusion liver preservation protocol

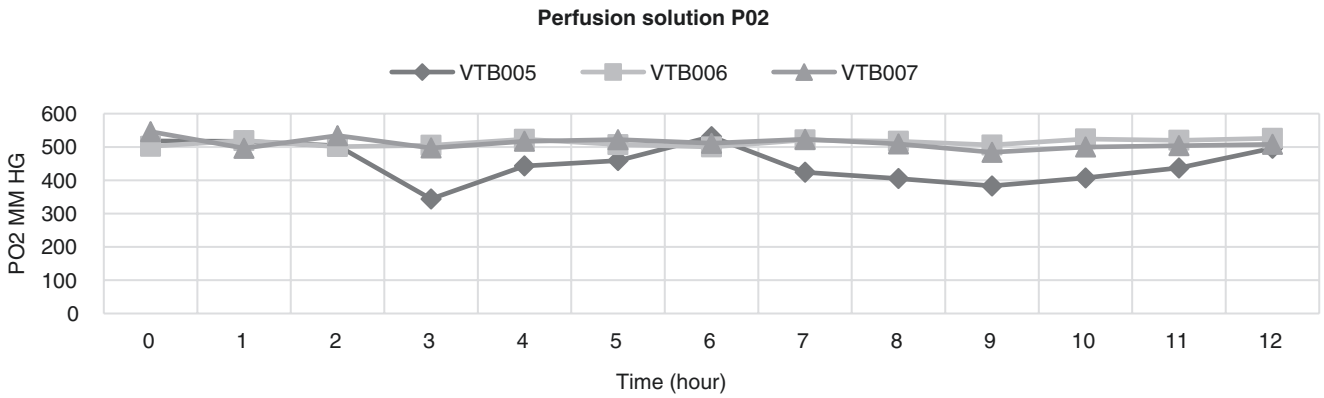


Fig. 39.4 Partial pressure of oxygen (pO_2 – mmHg) in the perfusion solution during the 12-hour machine perfusion liver preservation protocol

viously reported in hamsters that received in-vivo infusion of HBOC-201 (Hemopure), nitrite/nitrate ratios showed significantly lower values (baseline, <10× vs OxyBridge-infused, <100×) in the OxyBridge /MP experiments in pig liver (Fig. 39.4).

The presence of lactate would indicate anaerobic metabolism. The livers cleared lactate within the first 3 hours and the values were kept within normal values throughout the remaining perfusion time. This is another sign of effective ex-vivo oxygenation provided by the **OxyBridge** component (Fig. 39.5).

The pH was also sustained within normal range over the 12-hour period without the need of having $NaHCO_3$ infusions as seen in RBC-based systems (Fig. 39.6).

Mitochondrial function was depicted by Respiratory Control Ratio (RCR). Mitochondria were isolated from fresh liver biopsies by differential centrifugation and resuspended in Respiration buffer (120 mM KCl, 25 mM sucrose, 10 mM HEPES, 1 mM EGTA, 1 mM KH_2PO_4, 5 mM $MgCl_2$) for oxygen consumption measurements utilizing a Clark electrode. Respiratory control ratio was found by expressing State 3 (ADP dependent rate) over State 4 (succinate dependent rate). Mitochondrial function was analyzed in a sealed oxygraph chamber fit with a Clark-type oxygen electrode (Instech Laboratories Inc., Plymouth Meeting, PA, USA) connected to a data recording device (DATAQ Systems. The mitochondria efficiency was calculated by RCR, which measures the ratio of mitochondrial respiration supporting ATP synthesis to that required to offset the proton leak. RCR was normal and stable throughout the entire 12 hours f MP and oxygenation with the HBOC, revealing adequate oxidative phosphorilation by the tissues perfused with the HBOC (Fig. 39.7).

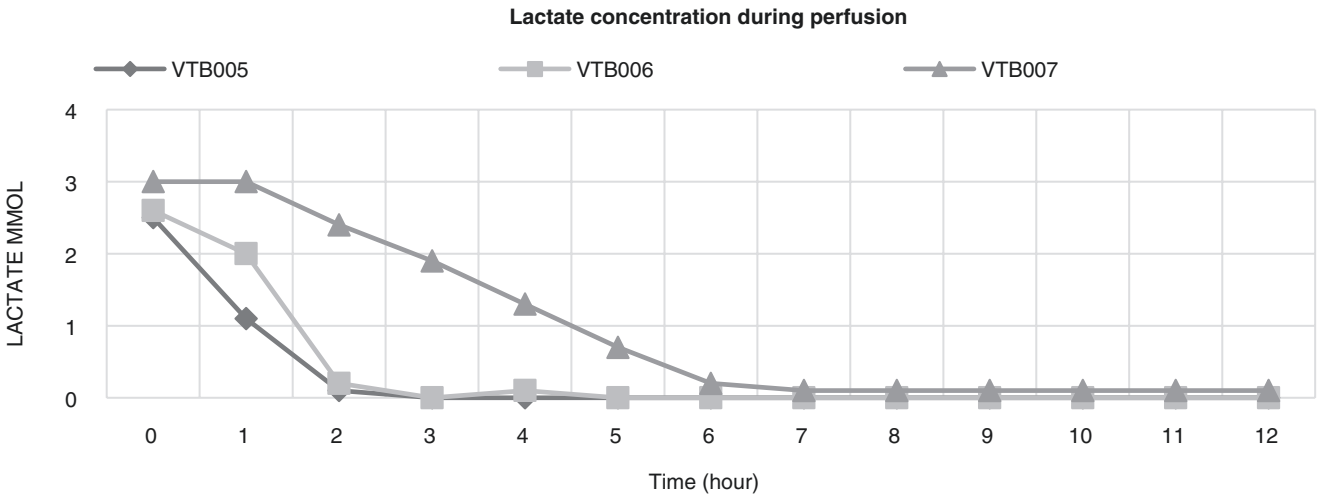


Fig. 39.5 Lactate concentration (mMOL) during the 12-hour machine perfusion liver preservation protocol

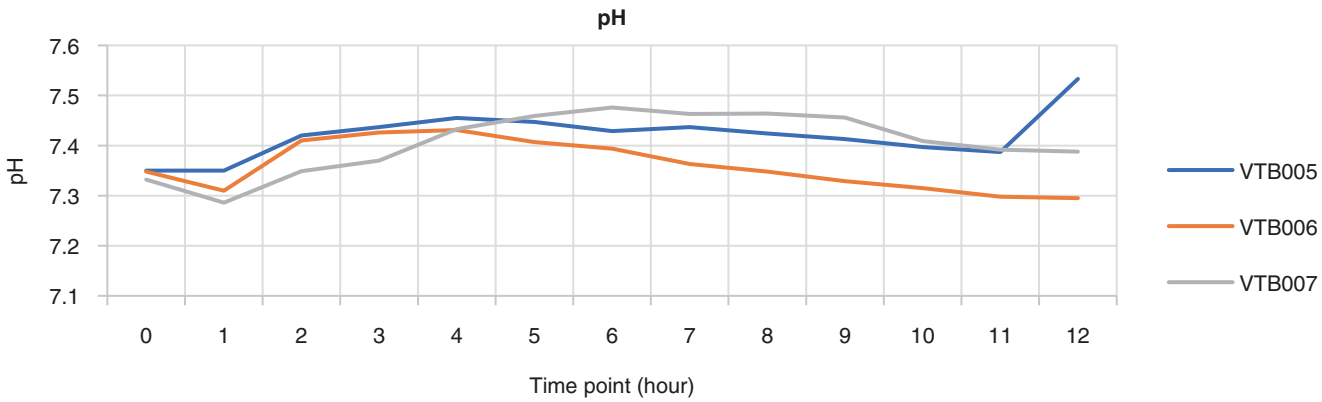


Fig. 39.6 pH range in the perfusion solution during the 12-hour machine perfusion liver preservation protocol

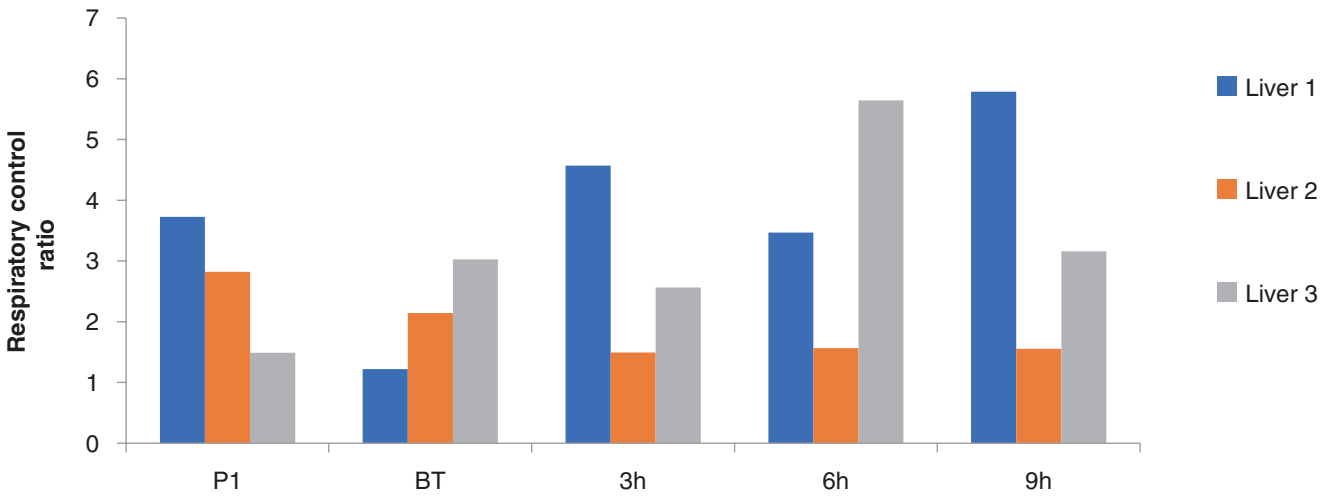


Fig. 39.7 Respiratory control ratio (RCR) from liver tissue during the 12-hour machine perfusion liver preservation protocol

Basal ATP generation rate was quantified to determine whether energetics were sufficient throughout the experiment. ATP generation was sustained throughout the entire duration of the experiments (12 hours).

Mitochondrial H_2O_2

Hydrogen peroxide (H_2O_2) generation was quantified in isolated mitochondria by measuring the rotenone sensitive oxidation of Amplex Red spectrophotometrically in the presence of nitrite, the nitrite reductase activity of deoxyhaemoglobin creates a balance between NO formation and haem-based NO scavenging that dictates the degree to which NO can stimulate signaling and vasodilation [26].

Additionally, mitochondrial hydrogen peroxide production was measured as an indicator of ROS generation in the tissue (a natural response to ischemia/reperfusion). Notably, hydrogen peroxide generation remained steady and did not increase (as would be expected to occur in injured tissue). The levels of ROS were low in spite the high levels of oxygenation (pO2 = 600 mmHg) experienced with the HBOC solution (Fig. 39.8).

Nitrite and Nitrate Measurements

Nitrite and nitrate concentrations were measured by reductive chemiluminescence. Briefly, nitrite was measured by injecting samples into a purge vessel containing a solution of tri-iodide, connected in line to a Nitric Oxide Analyzer (Sievers, GE). Nitrate was measured by injecting identical samples into a vessel purged with helium containing vanadium chloride to reduce nitrate to NO. The NO was detected by the Nitric Oxide Analyzer.

Our Nitrite/Nitrate data shows values significantly lower (baseline 10× lower and HBOC infused 100× lower) than the ones previously recorded in hamsters receiving in-vivo infusion of HBOC-201 (Hemopure) [24] (Fig. 39.9a, b).

Histopathology

The liver allografts sustained normal anatomical features throughout the entire experiment and had no signs of hypoxia and any other tissue damage during the 12-hour preservation. There were no signs of any damage to portal triads (hepatic artery, portal vein and bile ducts), hepatic sinusoidal system and central veins. There were no signs of edema and hepatic micro steatosis, which can be easily detectable within hours of low oxygenation to the hepatic tissue. Moreover, when compared to our previous experience with the bovine-derived (Hemopure) HBOC, there no signs of debris within the hepatic sinusoidal and complete integrity of the sinusoidal endothelial cells (SEC), which is by itself a major milestone in 12-hour liver preservation. Furthermore, electron microscopy (EM) assessment of the hepatocytes over this 12-hour period showed intact cell structures and preserved morphology of the mitochondria.

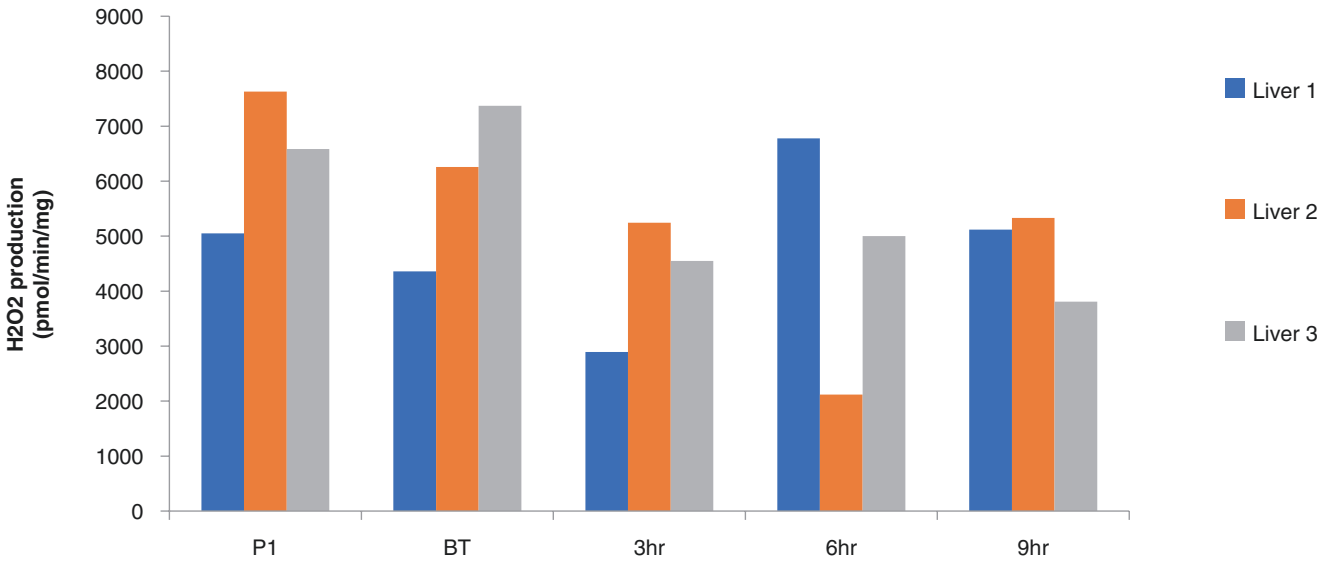


Fig. 39.8 H_2O_2 production (pmol/min/mg) of the hepatic tissue tissue assessed over a 12-hour period from serial fresh biopsies

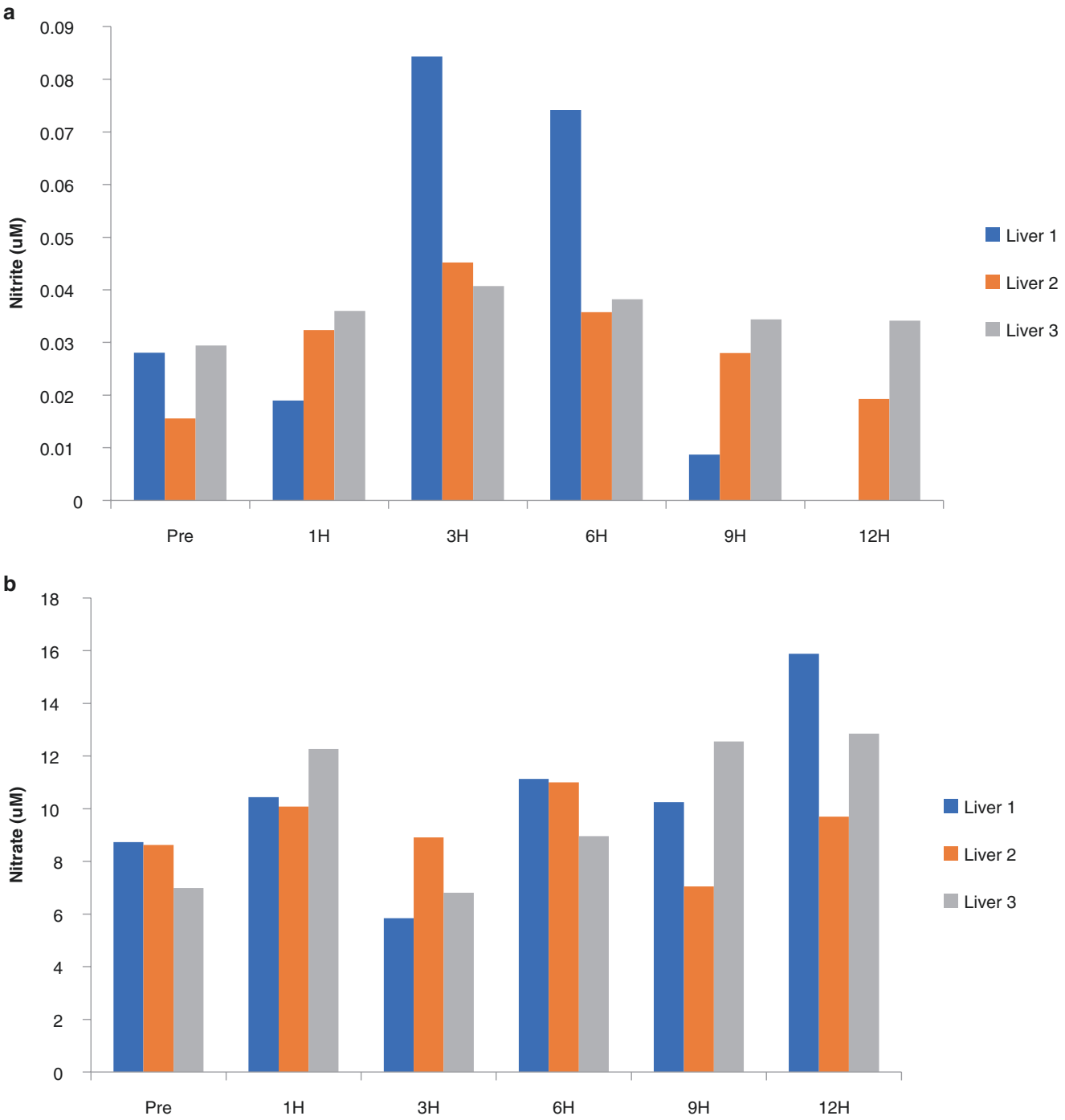


Fig. 39.9 (**a**) Nitrite levels (μM) produced by the liver tissue over a 12-hour period. (**b**) Nitrate levels (μM) produced by the liver tissue over a 12-hour period)

Conclusions from the Liver Experiments

1. The MP/HBOC preservation system was able to maintain full integrity and functionality of the liver allografts over a 12-hour period.
2. There were no signs of excessive auto-oxidation of the HBOC product. The methemoglobin levels were lower than the original data from in-vivo studies.
3. The levels of ROS were low in spite the high levels of oxygenation (pO2 = 600 mmHg) experienced with the HBOC solution.

4. There were no signs of vasoconstriction and hypertension. The machine perfusion parameters revealed progressive vasodilation in the arterial bed over the 12-hour period under sustained pulsatile pressures.
5. Despite a low hemoglobin concentration (3.5 g/dl) the oxygen delivery was eight times higher than oxygen consumption. Hepatocyte mitochondrial function was sustained over a 12-hour period in spite having a low Hb concentration (3.5 g/dl) in the perfusate.
6. The HBOC molecule showed sustained function (p50) and integrity (Hb content) over a 12-hour period.

Preliminary Pre-clinical Studies with Vascularized Composed Allotransplants (VCA) Utilizing the MP/HBOC Preservation System at 21 °C

These experiments were sponsored by the US DOD award (MR120034P4 – "*Ex-vivo* machine perfusion in composite tissue allotransplants (CTA) with a novel oxygen carrier system to enhance graft preservation and immunologic outcomes, 2013–15). This proof-of-concept large animal (swine) model for CTA was based on the heterotopic allotransplant of a vertical rectus abdominis myocutaneous (VRAM) flap with two dominant vascular pedicles into a fully mismatched recipient.

These experiments with the VRAM grafts were conducted in 2 separate stages, aimed to elucidate both the ex-vivo and the post-reperfusion in-vivo impact of prolonged preservation (14 hours) with a machine perfusion (MP) system using a newly developed hemoglobin-based oxygen carrier (HBOC) solution as the main perfusate. The tissue preservation protocols were conducted at 21 °C under sterile conditions and a full assessment of the biological modifications of the VRAM) grafts (e.g., histology, immunohistochemistry, inflammatory markers, and metabolomics) was conducted. Subsequent integrative analysis was performed with primary component analysis (PCA), dynamic Bayesian networks (DBN) inference and dynamic network analysis (DyNA). This has been an innovative approach to outline the biological implications of effective and prolonged ex-vivo oxygenation under subnormothermic (21 °C) conditions.

Rationale and Unmet Clinical Needs for VCA

There have been over 95 upper extremities and 26 craniomaxillofacial vascularized composite allotransplants (VCA) performed around the world [27]. Unfortunately, the growth of the VCA field has been slower than initially expected, largely due to the limited techniques currently available for graft preservation and due to the major immunological barriers faced after transplantation, where non-life saving procedures can lead to a lifetime burden of complications related to aggressive immunosuppressive therapy [28].

Recent studies indicate that more than 28,000 potential organ donors die each year in the United States [29]. However, a major obstacle to organ and tissue transplantation today remains the limited supply of suitable donors and within a feasible geographic location. VCA programs in the US have come recently under United Network for Organ Sharing (UNOS) jurisdiction, which led into further oversight for all regulatory aspects of transplantation and graft allocation [30]. In VCA, unlike internal organs, matching of skin color, tone, gender and size of the graft in addition to blood type between donor and recipient imposes additional operational and logistic limitations that can significantly restrict graft allocation and transplantation within suitable matches [31]. The need for organ and tissue sharing across a wider geographic distance imposed by the national donor allocation system managed by the Organ Procurement and Transplantation Network (OPTN) has implicated in extended cold ischemia time (CIT) for the VCA community [32].

The implementation of federal policies for VCA allocation initially intended to enhance recipient matching has extended sharing across wider geographic areas, which might implicate on unacceptable CIT for several donor-recipient combinations separated by longer distances.

Cold static preservation (CSP) has been the standard of care for organ and tissue preservation for almost 40 years [11]. Cold ischemic injury can induce irreversible perturbations in osmoregulation, energetics, and aerobic metabolism. In VCA, CSP induces a progressive reduction in interstitial oncotic pressure, allows interstitial expansion and edema and leads to further capillary compression and tissue injury [33]. Ischemia reperfusion injuries (IRI) have received further attention in VCA, where injury patterns in striate muscle demonstrate parallels to cardiac myocytes experiencing ischemic insults [34]. In VCS, both muscle and adipose tissue have elevated metabolic rates and are therefore more susceptible to damage during extended preservation and after reperfusion [35].

The heterogeneous composition of the VCA grafts can be further exemplified by the presence of viable skin (superficial) being found in musculocutaneous autografts, in spite the presence of significant necrosis within the muscular tissue immediately following transplantation [36]. The military population has been significantly affected by devastating limbs and soft tissue injuries when experiencing major trauma in the battlefield. Over 87% of all injury mortality occurred in the premedical treatment facility (MTF) environment [28]. The injury focus in short term mortality was mostly related to physical dismemberment (32%), catastrophic brain injury (38%), and massive cardio- thoracic

injuries (24%) [30]. Destructive abdominopelvic injuries were responsible for more than 6% of these casualties.

The wars in Afghanistan and Iraq, the most prolonged military conflict in US history, resulted in over 40,000 extremity injuries and 2500 amputations. Limb loss from upper extremities (UE) arterial injuries ranges from 1% to 18%. UE injuries remain a major therapeutic challenge for early revascularization and subsequent soft tissue coverage. Mortality from UE has been recorded as high as 34% in recent series when combined to concomitant injuries.

These proof-of-concept pre-clinical studies in a large animal model (swine) were primarily designed to define the safety and the efficacy of machine perfusion preservation in VCA over a 14-hour period when compared to cold storage (CS) as the current standard of care. We also aimed to establish some mechanistic pathways for early inflammatory markers, while pursuing the subsequent metabolic impact of prolonged preservation followed by transplantation by conducting extensive metabolomics analysis. To establish reliable clinical correlations, the preservation experiments were followed by graft implantation and a seven-day postoperative follow up before an end-study necropsy.

Methods

The surgical model for VCA was based on the heterotopic allotransplant of a vertical rectus abdominis myocutaneous (VRAM) flap with two dominant vascular pedicles into a fully mismatched recipient (Fig. 39.10).

These experiments with the VRAM grafts were conducted in two separate stages, aimed to elucidate both the ex-vivo and the post-reperfusion in-vivo impact of prolonged preservation (14 hours) with a machine perfusion (MP) system using a newly developed hemoglobin-based oxygen carrier (HBOC) solution as the main perfusate. Briefly, *ex-vivo* experiments with the VRAM grafts were conducted over a 14-hour preservation period at 21 °C. VRAM grafts were initially recovered under strict surgical techniques for tissue transplantation, meaning meticulous and bloodless dissection followed by isolation of both the arterial and venous vascular pedicles. No nerve reconstruction was performed. The VRAM grafts were subsequently preserved by two different modalities:

1. study group – MP/HBOC (n = 4);
2. control group – cold storage preservation (CSP) (n = 4).

The MP protocol of the VRAM grafts was performed with a prototype Liver Assist Device®, Organ Assist, Groningen, Netherlands in combination with our proprietary combination of HBOC solution with a HES-based colloid developed initially at University of Pittsburgh and further licensed by Virtech Bio Inc. (WO2014059316A1, PCT/US/2013/064607) [37]. The Liver Assist Device was further modified to accommodate a single arterial infusion port within a new set of pressures and flow. The perfusion chamber was modified and upgraded to accommodate a new medical PVC mesh designed to support the VRAM) graft in a horizontal position while allowing free venous and lymphatic drainage across the mesh.

For these experiments, the starting hemoglobin level as measured by an ABL800flex (Radiometer, Copenhagen) blood gas analyzer was 3.5 g/dL. The baseline settings for the MP system were: 60 mmHg pressure, 21 °C, FiO2 60%, sweep gas 0.3 L/min. Perfusion was begun at a pressure of 60 mmHg with a pulse frequency of 1 Hz, achieving a flow rate of 10 mL/min. The initial saturation level of the blood gas was 93 percent. HBOC solution at a pO_2 of ~400 mmHg. The MP device alters centrifugal pump speed to maintain a set pressure. After 2 hours, with flows exceeding 25 ml/min, the pressure set point was lowered to 50 mmHg where it was maintained throughout the remainder of the perfusion (Fig. 39.11).

After 14 hours, the VRAM) graft was removed from the MP device, weighted, and processed for additional studies. The subsequent *in-vivo* studies (Stage 2) were also composed of 2 groups of 4 animals each. Both groups (MP, n = 4 and CSP, n = 4) received heterotopic (cervical implantation) VRAM allografts after a period of 14 hours of preservation.

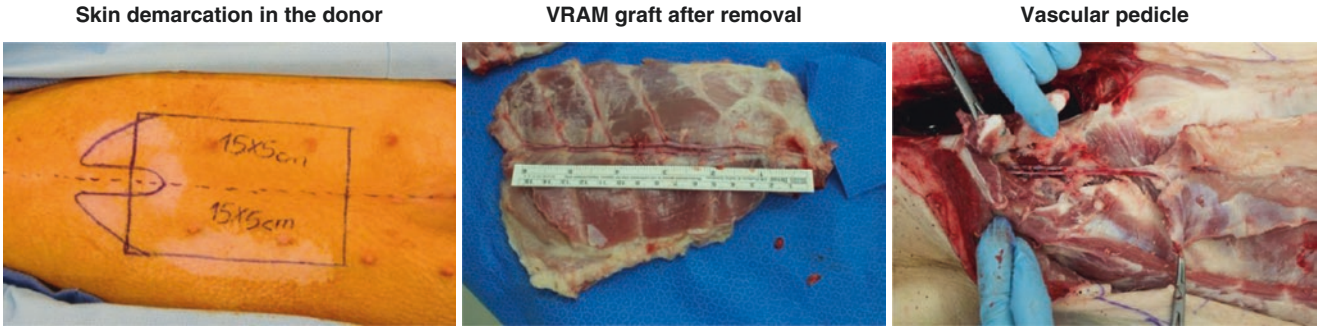


Fig. 39.10 Skin demarcation in the donor, VRAM graft, and vascular pedicle

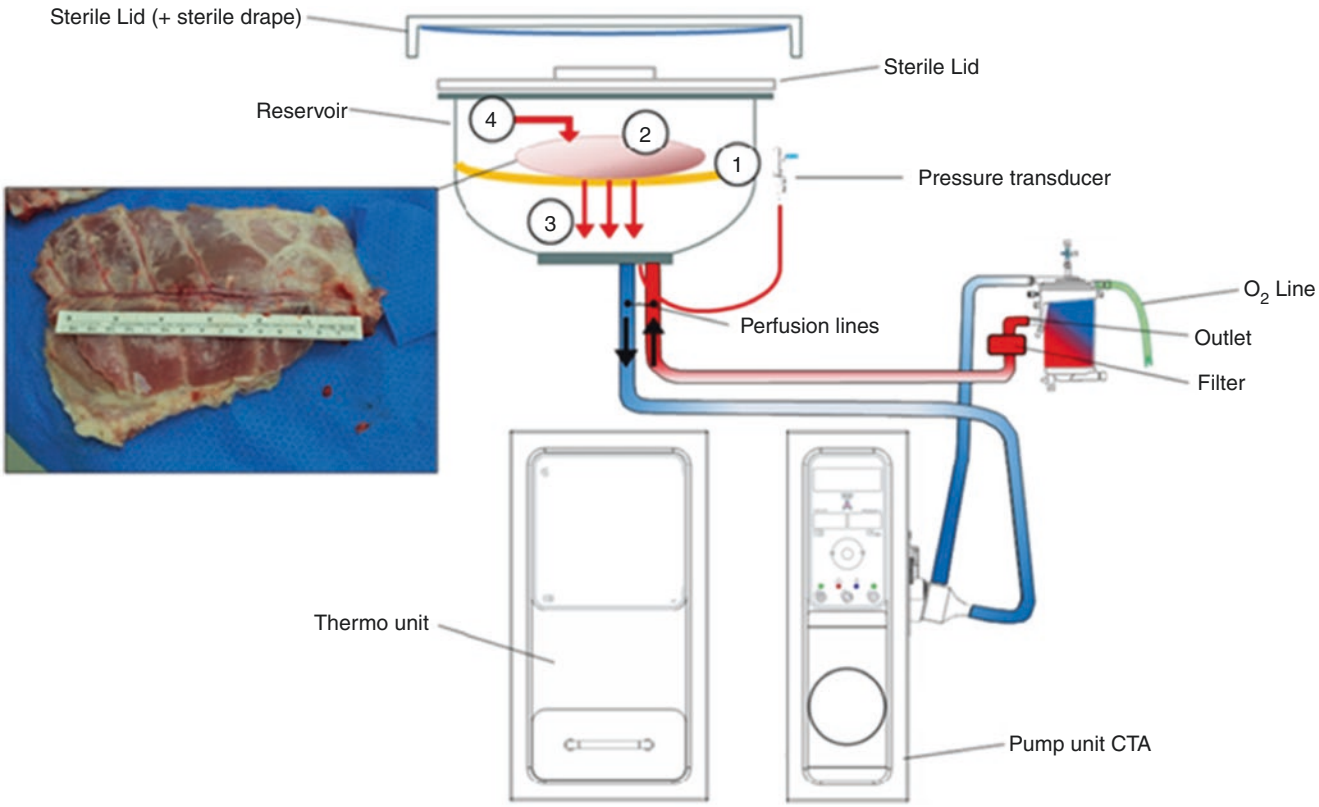


Fig. 39.11 Machine Perfusion device to preserve the VCA utilizing a single arterial infusion port

All animals (donors and recipients) were placed under general anesthesia and fully monitored. All procedures were performed under sterile conditions after being approved by the IACUC, U Pitt. The VRAM grafts were recovered en-bloc from an abdominal approach, where the vascular pedicle was fully isolated prior to systemic heparin infusion (20,000 U) and cross-clamp. The VRAM graft was removed from the field and immediately flushed (20 ml) with cold (4 °C) lactate ringer (LR) after arterial cannulation was completed (18Fr). A brief backtable procedure was conducted, where baseline biopsies were obtained prior to VRAM graft weight measurements. The CS group grafts were flushed with additional 100 ml of UW and packed (double bag) prior to placement into a temperature-controlled ice chest. The MP grafts were placed into the MP device, where the arterial cannula was immediately connected to the arterial infusion port. The MP preservation protocol was started and ABG samples from the perfusate were obtained every 15 minutes for the first hour and every hour for the remaining 13 hours.

After the completion of the preservation protocol, the VRAM grafts were implanted in an upside-down vertical orientation in the left cervical region in both groups, following a left cervicotomy and a meticulous dissection of the vasculature (carotid artery and jugular vein). The donor epigastric artery was anastomosed in an end-to-end fashion with interrupted 8-0 Prolene sutures to the recipient left carotid artery and the donor epigastric vein was anastomosed in an end-to-end fashion with interrupted 8-0 Prolene sutures to the recipient left jugular vein. The VRAM grafts were fully reperfused after the completion of the vascular anastomosis. The grafts were further secured without any vascular traction in the cervical region with additional subcutaneous sutures before the final skin closure with staples. A permanent central venous access was instituted by placing a Broviac catheter (BC) into the right superior vena cava after the completion of the transplant procedure. This was achieved by a right cervicotomy followed by cannulation of the internal jugular vein and exteriorization of the BC in the right interscapular space through a subcutaneous tunnel. The animals were extubated in the operative room and kept in an intensive care unit for 24 hours. The animals were subsequently transferred into a single room where they recovered for 6 additional days under strict surveillance. All the animals received 1 g of Solumedrol IV in the operative room before VRAM) graft reperfusion and triple immunosuppressive therapy after transplantation composed by Tacrolimus, Mycophenalate Mofetil and Prednisone. The animals had daily clinical and laboratorial assessment. Additional studies (histology, inflammatory markers and metabolomics) were performed to assess graft viability and the impact of ischemia reperfusion injuries (IRI) suffered after this prolonged period of preservation. The VRAM grafts were biopsied on

days 2, 4 and 7 after light IV sedation was provided and under sterile surgical technique with punch biopsy devices. To assess the potential degree of myolysis from IRI and the level of VRAM damage after 14 hours of preservation, myoglobin levels were measured daily in the recipient's peripheral blood after transplantation over a 7-day period.

Histological Analysis

All biopsies' samples from the VRAM grafts were fixed in 10% buffered formalin, embedded in paraffin, sectioned (5 μm) and stained with hematoxylin and eosin for histological analyses. The severity of VCA IR injuries was blindly graded by transplant pathologists initially using the International Banff Criteria [36]. The VRAM tissue obtained through punch biopsies contained skin, muscle, nerve, adipose and muscular tissue segments.

Inflammatory Markers

To quantify the inflammatory process triggered by IRI and the imminent alloreaction experienced by the VRAM after graft implantation, a full cytokine profile was obtained during VRAM graft preservation in both groups (MP and CSP). Subsequent samples were obtained from tissue biopsies during the postoperative period. Tissue and perfusate assays of interferon IFN-γ, IL-10, IL-12/IL-23 p40, IL-1β, IL-4, IL-6, IL-8 and tumor necrosis factor (TNF)-α were carried out using a Luminex™ beadset from Affymetrix (Santa Clara, CA). GM-CSF, IL- 1α, IL-1RA, IL-2 and IL-18 were measured using a Luminex™ beadset from Millipore (Merck KGaA, Darmsdadt, Germany). Tissue samples were normalized by protein content to account for experimental variability in cell number and protein concentration among individual samples.

Standard statistical analysis (ANOVA) of the cytokines' concentration across time points did not show any significant difference between the two groups during the ex-vivo stage. A subsequent integrated system analysis utilizing primary component analysis (PCA), dynamic Bayesian networks (DBN) inference and dynamic network analysis (DyNA) was able to demonstrate the different inflammatory pathways experienced by the two groups regarding the preservation method over a 14-hour period [38].

Subsequent integrative analysis was performed with primary component analysis (PCA), dynamic Bayesian networks (DBN) inference and dynamic network analysis (DyNA). This has been an innovative approach to outline the biological implications of effective and prolonged ex-vivo oxygenation under subnormothermic (21 °C) conditions.

Metabolomics

Tissue samples from the VRAM) grafts were obtained during preservation (0, 5, 9 and 14 hours) and after the transplant procedure on POD 0, 2, 4 and 7. These tissues were immediately frozen (OCT) and further submitted to Metabolon Inc., Raleigh, NC for metabolomics analysis. Samples were inventoried upon arrival and promptly kept at −80 °C [39]. At the time of analysis, Metabolon's standard solvent extraction technique was used to extract and prepare samples for analysis. For analysis on the GC/MS and LC/MS systems, the extracted samples were divided into equal portions. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers. The sample preparation process was carried out using the automated MicroLab STAR® system from Hamilton Company. Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions, one for analysis by LC and one for analysis by GC. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS. The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns.

Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% Formic acid, while the basic extracts, which also used water/methanol, contained 6.5 mM Ammonium Bicarbonate. The MS analysis alternated between MS and data dependent MS^2 scans using dynamic exclusion. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the Client Matrix samples, which are technical replicates of pooled client samples. There were 653 compounds analyzed. Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, Welch's two-sample

t-tests were used to identify biochemicals that differed significantly between experimental groups. To identify biochemicals that varied substantially across experimental groups, Welch's two-sample t-tests were performed. A false discovery rate (q-value) estimate was generated to account for the numerous comparisons that are common in metabolomic-based research. The *q*-value describes the false discovery rate; a low *q*-value ($q < 0.10$) is an indication of high confidence in a result. While a higher *q*-value indicates diminished confidence, it does not necessarily rule out the significance of a result [40].

Statistical Analysis

To define statistically significant differences in inflammatory mediators assayed in graft tissue during the period of preservation with MP /HBOC as a function of experimental group, we conducted standard statistical analyses of all data essentially as previously described [41]. Analyses were performed using SigmaPlot™ 11 (Systat Software, Inc., San Jose, CA), (MatLab® (MathWorks, Inc., Natick, MA), and StatView® (SAS Institute, Inc., Cary, NC). Comparisons of Luminex™ and qRT-PCR gene expression data were analyzed using ANOVA and the Tukey-Kramer *post hoc* test where appropriate. When only two comparisons could be made, an unpaired two-sided t-test was used. Experimental results were determined to be statistically significant when $p < 0.05$. Continuous variables were expressed as mean ± SEM. For the ANOVA outcomes, 80% power and 5% significance were used to find a 25% difference in outcomes. We expected to find statistically significant differences in inflammatory mediators in samples from graft tissue preserved with MP / HBOC vs. control.

To define principal drivers of inflammation in graft tissue during the preservation period with the MP /HBOC system as a function of experimental group, we carried out PCA of all data essentially as we described recently. The goal of this analysis was to identify the subsets of inflammatory mediators (in the form of orthogonal normalized linear combinations of the original mediator variables, called principal components) that were most strongly correlated with a given experimental procedure or outcome, and that thereby could be considered principal drivers of each response.

To perform this analysis, the data was first normalized for each mediator (i.e., a given value divided by the maximum value for a given inflammatory mediator), so that all mediator levels could be converted into the same scale (from 0 to 1). Thus, any artefactual effects on variance caused by the measured concentration ranges for various cytokines were removed. Only components accounting for at least 70% or 95% of the variation in the data were evaluated. Each cytokine's coefficient (weight) was multiplied by the eigenvalue associated with each of these leading major components. This product quantified the contribution of a certain inflammatory mediator to the variation explained by a particular main component. The overall score given to each cytokine/chemokine was the sum of its scores in each component. This gave us a measure of a cytokine's contribution to the overall variance of the system. We expected to find different inflammatory drivers in graft tissue preserved with MP / HBOC vs. control.

Dynamic Bayesian Network (DyBN) inference and Dynamic Network Analysis (DyNA) were used to define networks of cytokines and chemokines model the evolution of the probabilistic dependencies within a system over time. DyBN inference were carried out using MATLAB™ (The Math Works, Inc., Natick, MA), using an algorithm adapted from Grzegorczyk & Husmeier[2] and revised recently by the Vodovotz group [42]. DyBN allowed us to assess the dominant inflammatory mediators and the probable interaction among various mediators, including possible feedback loops. DyNA is an algorithm developed by the Vodovotz group using MATLAB™; though this algorithm cannot show feedback loops, it does highlight correlations across individual time intervals, allowing for a representation of the connectivity among inflammatory mediators as a function of time [35]. We expected to find different inflammatory networks in graft tissue preserved with MP-BMPS/HBOC vs. control, since we detected significant differences between the two groups after the initial clinical, histological and metabolomics analysis.

Results

The MP/HBOC system provided low pressures (50–55 mmHg), low flows (20–80 ml/min) and full oxygenation ($FiO_2 = 60\%$ @ 400 ml/min) to the VRAM grafts (Fig. 39.12).

The MP/HBOC system stabilized the perfusate's pH (7.55–7.6) while keeping lactate levels under 4 mmol/L (Fig. 39.3). In spite the prolonged period of perfusion (14 hours), there was no need to proceed with additional $NaHCO_3$ infusions as seen in previous similar experiments with different MP systems (Fig. 39.13).

There were no technical complications from any of the surgical procedures and the animals recovered well from transplantation under strict USDA guidelines. Myoglobin blood levels were significantly higher in CSP flap recipients, showing the magnitude of the IRIs seen in the current standard of care for tissue preservation when compared to the MP/HBOC system (Fig. 39.14).

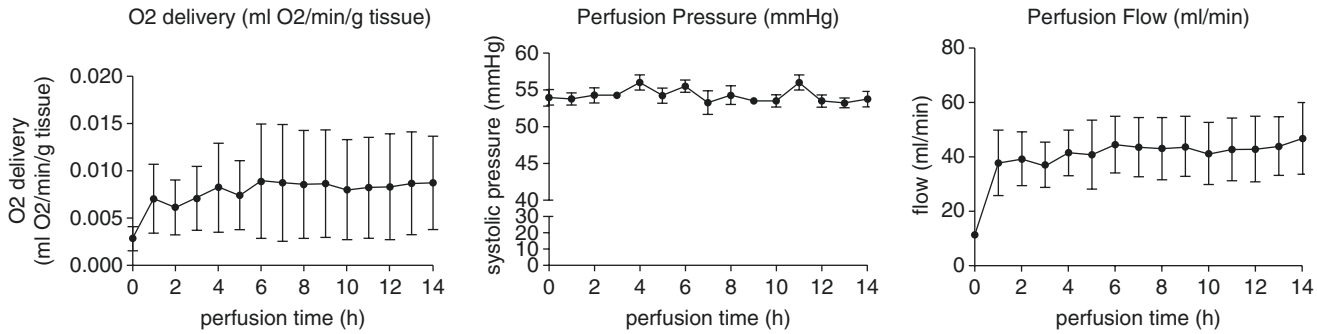


Fig. 39.12 Perfusion parameters (O_2 delivery, Perfusion pressure and flow) for VCA preservation over a 14-hour period

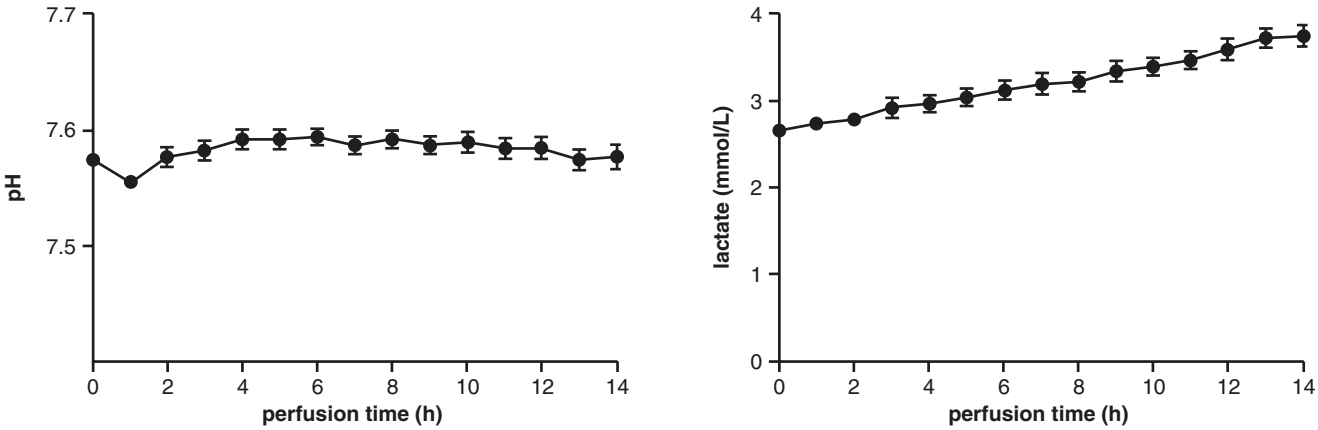


Fig. 39.13 Biochemical features (pH and Lactate concentration) of the perfusate of the MP/HBOC system over a 14-period

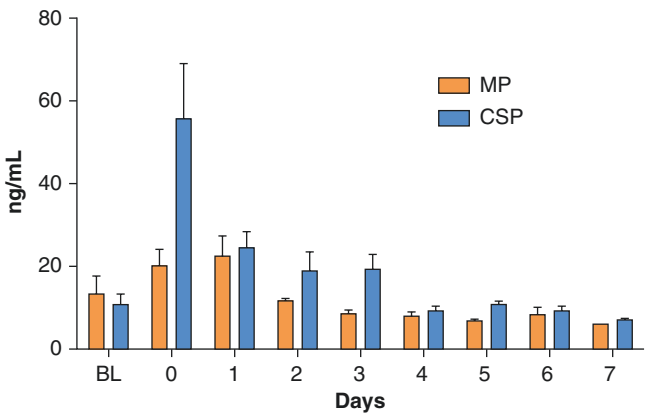


Fig. 39.14 Myoglobin levels in blood samples from the recipients of allografts preserved be either Machine Perfusion (MP) or Cold Storage Preservation with UW (CSP)

Clinical and Histopathologic Analysis

Initial histological assessment of buffered formaldehyde-fixed paraffin-embedded biopsies of skin, muscle, nerve, adipose and muscular tissue was performed by a transplant pathologist (H&E staining). The VRAM allografts were biopsied every 4 hours during preservation and at days 2, 4 and 7 after transplantation. Early damage on the CSP grafts was clearly detected within the initial samples (4 and 8 hours). The presence of early hypercontracted sarcomeres (contraction bands – CB) and frequent sarcolemmal ruptures that gave rise to the term "contraction band necrosis" were observed in the sarcomeres, which were subsequently followed by moderate to severe IRI in the CSP flaps *in vivo*. CB are thick, irregular, transverse eosinophilic bands in necrotic myocytes. The bands are small groups of hypercontracted and disorganized sarcomeres with thickened Z lines. The sarcolemma is disrupted, and the mitochondria located between the CB swell. CB occur whenever there is a massive influx of Ca^{++} into the myocytes. The CB became more prominent after reperfusion and led into extensive necrosis within the CSP group. Adiponecrosis and skin necrosis were also significantly higher in the CSP group.

Post-reperfusion Findings

The VRAM grafts reperfused well and had no signs of early technical problems. Figure 39.9 shows the clinical aspects of the tissues after transplantation. Pen-Rose drains were utilized to avoid fluid collection within the subcutaneous tissue

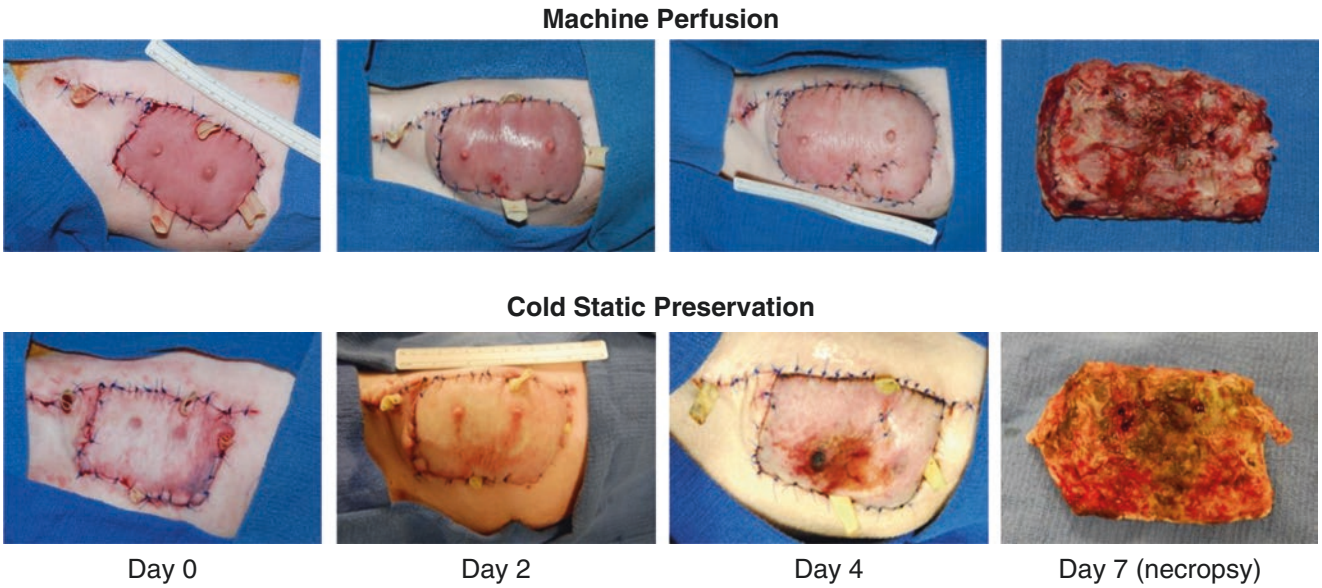


Fig. 39.15 Macroscopic view of the VCA after the initial surgical implantation (day 0) in the recipients' cervical area and over the postoperative period (days 2, 4 and 7)

that could compress the vasculature. The VRAM grafts from the MP/HBOC group were effectively oxygenated over a 14-hour period at 21 °C. There were clear signs of vasodilatation within the graft, which re-perfused well after implantation, contrary to the CSP grafts, which were cold and vasoconstricted (Fig. 39.15).

The skin portion of the VRAM grafts was mildly affected on both groups initially, which was disproportional to the lesions documented in the deeper layers (adipose and muscle) over the postoperative period. The control CSP group progressed towards further necrosis within the adipose and muscle tissues, while showing scattered ischemic ulcerations within the skin. There were no clinical and histological abnormalities on the skin portion of the VRAM grafts from the MP/HBOC group. There were significant differences between the two groups (CSP and MP) when overall viability and full thickness tissue integrity was assessed for IRI. The MP group had mild signs of IRI in the 3 segments of the graft (e.g., skin, adipose tissue and muscle). The CSP group had moderate to severe signs of IRI within the 3 segments of the VRAM grafts. There were also signs of considerable irreversible damage within the vascular endothelial cells leading to further apoptosis and necrosis within the adipose and muscular tissues in the CSP group. The presence of perivascular edema, red blood cell extravasation, leukocyte adhesion and infiltration, intraluminal thrombi of microvasculature and progressive loss of the endothelial cell layer in mid-size vessels were also observed in the CSP group. The initial endothelial cell dysfunction apparently led to further vascular leakage in the CSP group. Additional macrophage and eosinophilic infiltration were detected in the CSP group. Nuclear changes (e.g., pyknosis, karyorrhexis and karyolysis) were further observed as signs of apoptosis and necrosis within the muscular layer in the CSP group. Furthermore, fiber disruption, loss of striation and additional decomposition of both the endomysium and epimysium were clearly noticed in the CSP group. These classic histological inflammatory features of significant IRI injuries were rarely noticed in the MP group. Figure 39.10 displays a comparison chart of the surgical wound and tissues obtained through punch biopsies from both groups (MP on the left and CSP on the right panel) at the second postoperative day. The degree of apoptosis and necrosis is significantly higher in the CSP group than the MP group, where intraseptal lymphocyte infiltration and progressive muscle fiber atrophy is also seen. Cell debris (from resident cell populations and infiltrating leukocytes); proteinaceous fluid containing fibrin, fewer macrophages and occasional lymphocytes and/or plasma cells are seen in the CSP. None of these changes are seen in the MP/HBOC group.

A comparison of the tissues obtained through punch biopsies from both groups (MP on the left and CSP on the right panel) during the postoperative period shows the progressive degree of ischemia leading to irreversible necrosis in the CSP group. The CSP displayed a higher degree of inflammation, with clear signs of myofiber necrosis, myopathic changes in addition to edema and/or hemorrhage. Progressive lymphocyte infiltration and areas of hemorrhage were seen. The loss of muscle fibers and hypereosinophilic degenerative fibers were also seen in the CSP. Progressive granulomatous inflammation is seen among several aggregated, large, activated macrophages, epithelioid macrophages, or multinucleated giant cells The MP/HBOC group had muscle tissue with normal histological features.

At termination of the study (euthanasia on the 7th POD), the MP/HBOC VRAM grafts had normal macroscopic features while clear signs of extensive tissue necrosis within the subcutaneous tissues were seen in the CSP group. The MP/HBOC graft showed full vessel patency and no signs of necrosis within the deep portions of the graft. The degree of necrosis and overall tissue damage was significantly higher in the CSP group. There were extensive necrotic areas with moderate to severe amount of inflammation. Progressive neutrophilic infiltration led to necrosis and loss of muscle fibers. Extensive macrophage infiltration was observed leading into granulomatous-like changes resembling pseudo-abscesses surrounded by epithelioid macrophages, or multinucleated giant cells were seen in the CSP, where extensive muscle fiber degeneration and mineralization within multinucleated giant cells were present. The MP/HBOC group had muscle tissue with normal histological features. The MP/HBOC graft showed full vessel patency and no signs of necrosis within the deep portions of the graft. The control CSP group progressed towards progressive tissue necrosis and ischemic ulcerations of the skin, whereas the study MP group stabilized. There were significant differences between the 2 groups (CSP and MP) when overall viability and full thickness tissue integrity was assessed for IRI. The MP group had mild signs of IRI in the 3 segments of the graft (e.g., skin, adipose tissue, and muscle). The CSP group had moderate to severe signs of IRI within the 3 segments of the VRAM grafts. There were also signs of considerable irreversible damage within the vascular endothelial cells leading to further apoptosis and necrosis within the adipose and muscular tissues in the CSP group. The presence of perivascular edema, red blood cell extravasation, leukocyte adhesion and infiltration, intraluminal thrombi of microvasculature and progressive loss of the endothelial cell layer in mid-size vessels were also observed in the CSP group.

The initial endothelial cell dysfunction apparently led to further vascular leakage in the CSP group. Additional macrophage and eosinophilic infiltration were detected in the CSP group. Nuclear changes (e.g., pyknosis, karyorrhexis and karyolysis) were further observed as signs of apoptosis and necrosis within the muscular layer in the CSP group. Furthermore, fiber disruption, loss of striation and additional decomposition of both the endomysium and epimysium were clearly noticed in the CSP group. Punch biopsies from both groups at day 2 demonstrated increased apoptosis and necrosis in the CSP group than the MP group, with intraseptal lymphocyte infiltration and progressive muscle fiber atrophy. Cell debris (both from the resident cell populations and from infiltrating leukocytes); proteinaceous fluid containing fibrin, fewer macrophages, and occasional lymphocytes and/or plasma cells were seen in the CSP. None of these changes were seen in the MP/HBOC group.

Inflammatory Markers

To quantify the inflammatory process triggered by IRI and the imminent alloreaction experienced by the VRAM after graft implantation, a full cytokine profile was obtained during VRAM graft preservation in both groups (MP and CSP). We're able to demonstrate again, analogue to our previous liver experiments, a central role for TNF-α in the CSP group as major promoter for sustained and enhanced inflammation as demonstrated by the initial DBN inference (Fig. 39.16).

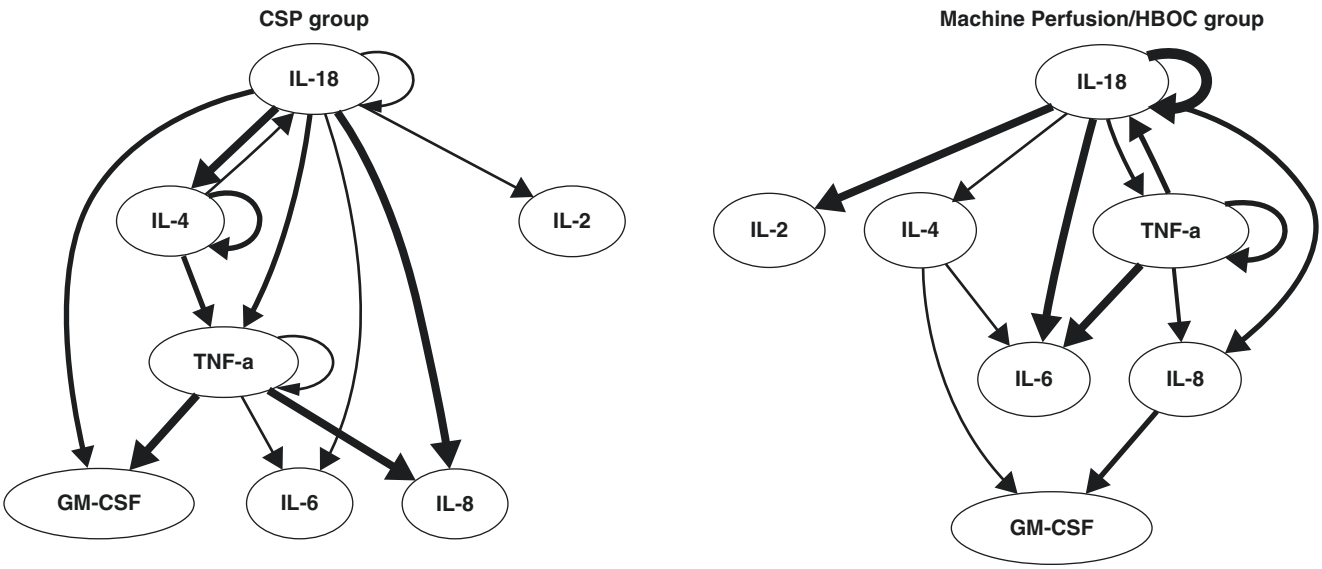


Fig. 39.16 Dynamic Bayesian Network (DBN) diagrams comparing the complexity of the inflammatory network between the MP/HBOC and the CSP groups

Subsequent dynamic network analysis (DyNA) showed a lower degree of complexity in the CSP inflammatory network in spite of sustaining a similar number of connections displays a horizontal analysis of all interactions observed among all the measured cytokines.

Network complexity scores are consistently higher in tissue networks than perfusate. For both sample types, the initial CSP network is the most complex, with CSP network complexity decreasing overtime. MP networks, which for both sample types start with lower complexity than CSP, remain relatively static in complexity throughout the preservation period (Fig. 39.17).

This analysis was extended to all horizontal interactions of the inflammatory markers in each time point. This analysis was also capable to reveal all positive (upregulation) and negative (downregulation) interactions among these inflammatory markers in a dynamic and rather precise manner. Furthermore, both groups (control and study) were able to be compared side-by-side in each time point and across the entire preservation period regarding the persistent interactions among all the inflammatory markers. This dynamic approach wasn't possible when standard statistical analysis was performed. Subsequent DyNA revealed rather interesting inflammatory pathways overtime in both groups. This modality showed a leading role in TNF-α activation seen earlier in the control group, where subsequent INF-ɣ activation was also detected.

This aggressive inflammatory pathway was very different than the one seen contemporaneously in the MP group, where a much simpler cytokine network was primarily driven by IL-4 and IL-6. Interesting enough, the histology analysis showed the early presence of contraction bands (CB) in the sarcomeres of the control group at the 4-hour mark, which remained anoxic and hypothermic for the entire duration of the experiment (14 hours). A similar histological pattern (CB) has been similarly described in the sarcomeres of the myocardium of patients with coronary artery disease in the period that precedes a well-defined ischemic myocardium infarct. Further analysis reveals the absence of any TNF-α in the MP group, showing the beneficial impact of effective ex-vivo oxygenation in this group. Previous clinical studies in patients with myocardium infarct (muscular damage from ischemia-reperfusion) have shown a major role in TNF-α and IL-1RA in the initial upregulation of the inflammatory process following myocardium damage from prolonged ischemia [43].

CSP and MP groups are compared. IL-18; IL-1b, IL-1RA and IFN-g are among the variables included in every tissue DyNA network. In the CSP analysis a complex initial network is followed by two networks in which a majority of variables only have two edges and whose structures are almost identical. Unlike CSP, the MP networks continue to fluctuate throughout the experimental time course.

Figure 39.18 shows a comparative assessment overtime of the complexity of the inflammatory networks. In spite moving towards a lower number of connections, the control group (CSP) yielded a more robust ischemia-reperfusion injury after the VRAM implantation into the recipients. This is a very important finding that reinforces the need for extended assessment of IRIs after graft reperfusion, rather than a brief ex-vivo assessment by blood flush as previously seen in additional studies [44].

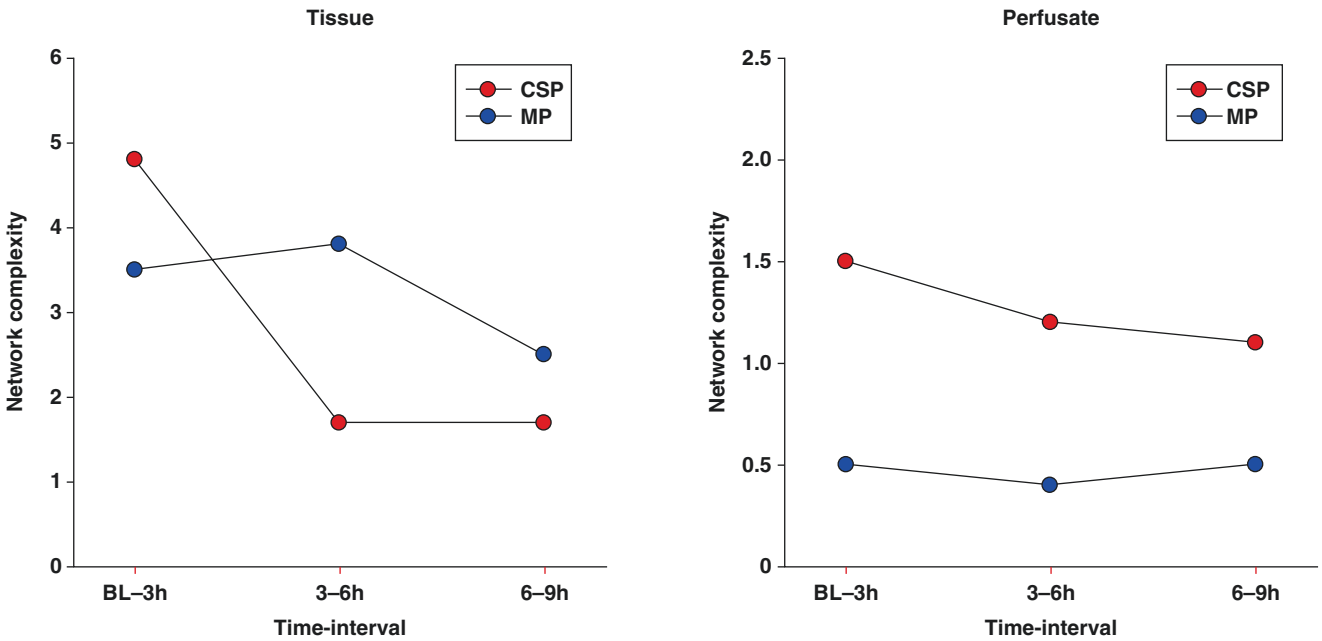


Fig. 39.17 Network complexity DyNA. *CSP* cold static and. *MP* machine perfusion perfusate

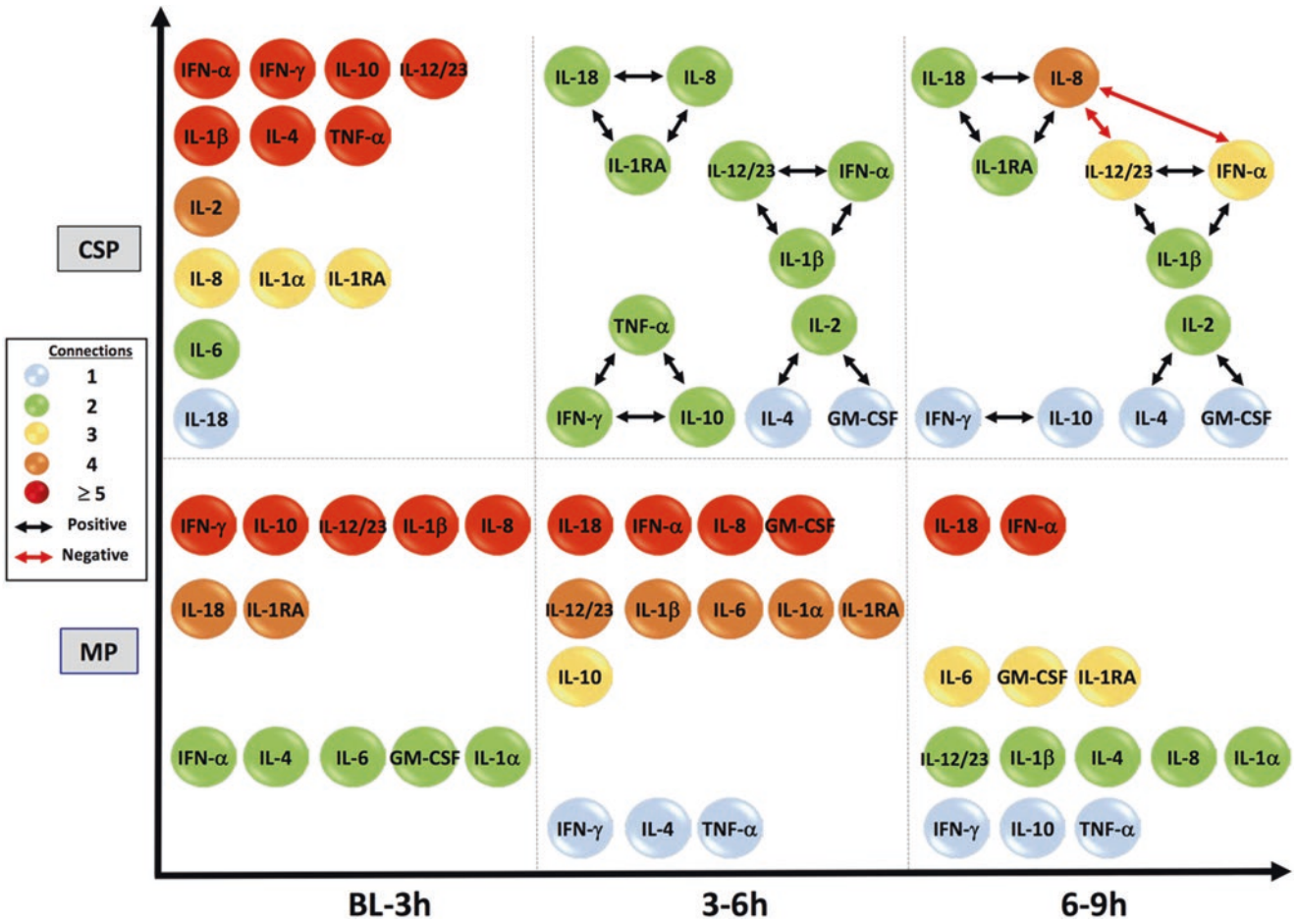


Fig. 39.18 Tissue sample DyNA networks suggest different network stability between preservation methods

When al data the data was combined and plotted in a dynamic fashion using DyNA tools, the control group showed an early upregulation of GM-CSF, which was followed by activation of both INF-ɣ and IL-2. These cytokines have been previously shown as potent mediators of early inflammation in patients undergoing controlled limb ischemia during elective procedures [45]. Further immune activation was provided by upregulation of IL-1a, IL-1b and IL-1RA, stemming by direct upregulation from IL-12, IL-10 and IL-8. Interestingly, the MP group showed a different inflammatory pathway in-vivo during the first 4 days [2–4], where GM-CSF appeared to downregulate the activation of the same cytokines (IL-10, IL-8 and IL-1b). Histologically, the amount of inflammation experienced by the MP group was significantly lower after VRAM implantation into the recipients. As we extended our follow up towards postoperative days 4–7, the MP group showed a sustained GM-CSF interaction with IL-12, IL-10 and IL-1a (intra VRAM cytokine levels). Histologically, the MP group showed decreased inflammation, absence of acute cellular rejection and progressive recovery from mild IRIs. On the contrary, the CSP group showed an intra VRAM) graft inflammatory pathway characterized by the absence of GM-CSF and INF-ɣ interactions, in a cytokine network with fewer overall connections when compared to the MP network. The CSP intra-graft profile appeared to sustain a close loop interaction between IL-1a and IL-1b, which leaked into additional activation of IL-12, Il-10, IL-8 and IL-6. Histologically, this inflammatory network was characterized by progressive muscle necrosis and a higher degree of early leukocyte infiltration.

There were clear signs of subsequent myofiber necrosis, myopathic changes in addition to edema and/or hemorrhage in the CSP group. Subsequent neutrophil infiltration and expanded areas of hemorrhage were clearly seen in the CSP group. The loss of muscle fibers due to hyper eosinophilic degeneration was significant. Progressive granulomatous inflammation evolved overtime among several aggregated, large, activated macrophages and epithelioid macrophages. There were extensive necrotic areas with moderate to severe amount of inflammation on day 7 in the CSP group. Progressive neutrophilic infiltration was seen around extensive necrosis and loss of muscle fibers. Late macrophage infiltration (day 7) was also observed, leading into

granulomatous-like changes resembling pseudo-abscesses surrounded by epithelioid macrophages, or multinucleated giant cells. This extensive muscle fiber degeneration evolved into further mineralization within aggregates of multinucleated giant cells.

Similar to the data from the ex-vivo stage, the complexity of the cytokine network appeared to have a bimodal behavior, where the level of complexity increased in the MP group overtime while the level of complexity decreased in the CSP over the 7 day period.

Metabolomics

The VRAM grafts in the MP system sustained an intact energetic metabolism fueled by glucose over the 14-hour period of preservation when compared to the CSP group. MP sustained normal skeletal muscle glycolysis as evident by higher: glucose 6-phosphate (↑23-fold, $p = 0.01$), fructose-6-phosphate (↑12-fold, $p = 0.03$), and phosphoenolpyruvate (↑8-fold, $p = 0.03$). Nucleic acid synthesis was significantly higher in the MP flaps: cysteine (↑3.88-fold, $p = 0.02$), ribose (↑22-fold, $p < 0.001$), ribonate (↑37-fold, $p < 0.001$), and ribitol (↑10.5-fold, $p < 0.001$). MP led to significantly higher levels of reactive oxygen species (ROS) scavengers in the VCAs: glutathione-cysteine disulfide (↑5.6-fold, $p = 0.01$) and N-acetylcysteine (↑40-fold, $p = 0.007$). Furthermore, SNMP provided sufficient energy precursors and metabolites: adenine (↑129-fold, $p = 0.002$), cAMP (↑3.7-fold, $p = 0.02$), AMP (↑3.2-fold, $p = 0.01$) and 3'-AMP (↑2.3 fold, $p = 0.01$). CSP grafts faced extensive amino acid metabolism dysregulation as suggested by significantly higher levels of: N^6-succinyladenosine ($p < 0.01$), valine ($p < 0.01$), 2-methylbutyrylcarnitine ($p = 0.01$), 3-hydroxyisobutyrate ($p = 0.01$), and ethylmalonate ($p = 0.009$) tissue levels. Glycogen reserves were higher in the MP group. There was adequate glucose supply in the MP and no signs on glycogen breakdown. The pentose metabolites were significantly higher in the MP group, showing a higher anabolic state when compared to the CSP group. The CSP group appeared to have a sustained catabolic state when compared to the MP group. There were signs of higher production of nucleotides and nucleic acids precursors in the MP. As previously seen in our experience with liver allografts under the MP/HBOC system, there was a significant (30-fold higher) increase in the metabolic pathways related to cell regeneration once oxygenation was effectively provided ex-vivo during preservation. There were signs of higher production of aromatic amino acids in the MP group when compared to the CSP group. The MP/HBOC system provided more effective antioxidant pathways when compared to the CSP group. There were higher levels of end-products from oxidized stress in the MP group, which can be seen as an indirect sign of lower stress from less significant IRIs when compared to the CSP group.

Contrary to our previous experience with livers, the VRAM) grafts under the MP/HBOC system showed lower fatty acid β-oxidation when compared to the CSP group. This means a lower of fatty acids into the mitochondria as a source of fuel. This also favors our initial findings regarding the preferential pathway for glucose as the primary source of energy in striated muscles. Further analysis of the purine metabolism (adenine components) showed indirect signs of higher ATP production in the MP group when compared to the CSP. The adenine family has a variety of roles in cellular respiration and protein synthesis. There were higher levels of cAMP in the MP group, showing higher ATP production in this group. AMP is used as a monomer in RNA synthesis. The cAMP as a derivative of ATP has a significant role in signal transduction. SNMP increased fatty acids (FAs) Ω-oxidation pathway as evidenced by significantly higher tissue levels of dicarboxylic FAs: 2-hydroxyglutarate (↑4.8 folds, $p = 0.02$), adipate (↑5.55 folds, $p = 0.003$), and 2-hydroxyadipate (↑2.5 folds, $p = 0.01$). Reduction of β-oxidation was evident by substantial increase in the levels of acyl-carnitine metabolites in the SNMP/HBOC grafts: cis-4-decenoyl carnitine (↑8 folds, $p = 0.04$), laurylcarnitine (↑8.3 folds, $p = 0.02$), oleoylcarnitine (↑9.5 folds, $p = 0.01$), myristoleoylcarnitine (↑22 folds, $p = 0.006$), and adipoylcarnitine (↑7 folds, $p < 0.001$). This was mirrored by significantly higher levels of end-products of β-oxidation pathway in the CSP group as shown by 4-hydroxybutyrate ($p < 0.01$). Cellular membrane integrity was well preserved histologically and further evidenced by significantly higher levels of phospholipids in the SNMP/HBOC group: oleoylcholine (↑5 folds, $p < 0.01$) and choline (↑3 folds, $p = 0.01$). In addition, early signs of myopathy were observed with significantly higher tissue levels of butyryl-carnitine ($p = 0.04$) in the CSP grafts, which were further corroborated by histopathologic analysis (Fig. 39.19).

Discussion

This extended preservation period in a pre-clinical large animal model (swine) appeared to induce significant oxidative cell damage in the CSP group. Well established cytokine pathways (e.g., TNF-α and IL-1 upregulation) were initially seen in the CSP group. Histologically, significant neutrophil followed by macrophage infiltration were extensively documented in the CSP group, where significant muscle necrosis led to irreversible muscle fiber degeneration followed by diffuse mineralization surrounded by aggregates of multinucleated giant cells (pseudo-abscess like lesions). This subsequent

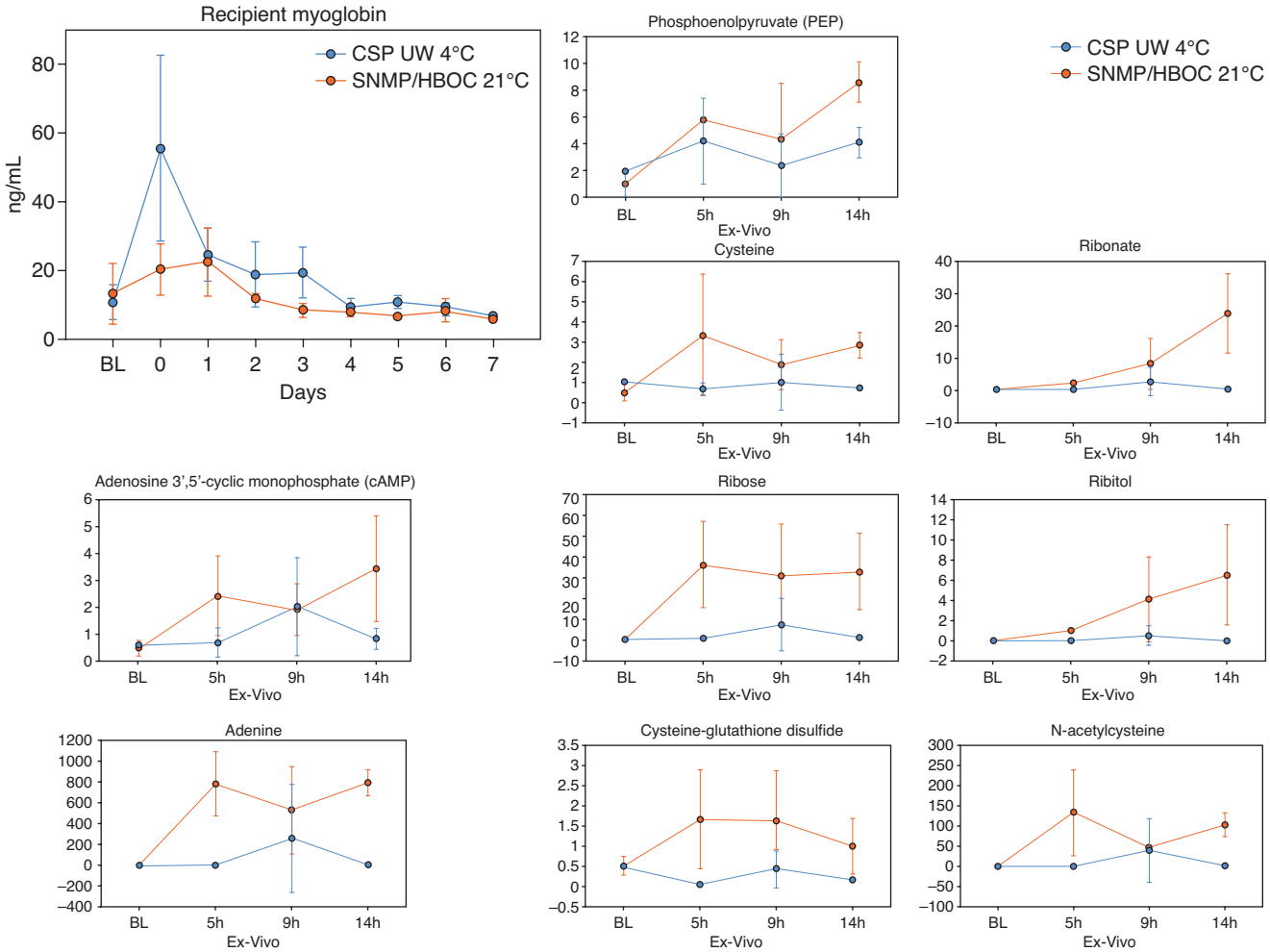


Fig. 39.19 Daily (base line (BL to 7) myoglobin levels (ng/ml) in the recipient's blood after the VCA transplants and selected metabolomics (phosphoenolpyruvate, cysteine, ribonate, adenosine monophosphate, ribose, ribitol, adenine, cysteine-glutathione and N-acetylcysteine) isolated from the MP system's perfusate during preservation (BL to 14 hours) showing the differences between the CSP and the MP/HBOC groups)

dynamic analysis of the inflammatory markers using PCA, DBN and DyNA tools was able to establish a more reliable link to the significant differences between the two groups documented by the initial histological analysis [46].

The MP/HBOC system can effectively preserve VRAM allografts when compared to CSP. MP significantly mitigates IRI, which was manifested earlier within the first 4 hours in the CSP group. Effective *ex-vivo* oxygenation with HBOC decreases post-transplant inflammation in skeletal muscle fibers and upregulates regenerative metabolic pathways driving early recovery from IRI. There is a similar up-regulation of TNF-α in the CSP group, which is similar to our previous data obtained in liver allografts after a period of 9 hours of preservation. Effective ex-vivo oxygenation with the MP/HBOC system avoids the early (hours) formation of hypercontracted sarcomeres (CB) and the subsequent development (days) of myofiber necrosis, myopathic changes, edema and hemorrhage seen extensively in CSP as the current standard of care. The significant IRI observed in the CSP group yielded a significant hypereosinophilic sarcomere degeneration leading into irreversible loss of muscle fibers, followed by progressive granulomatous inflammation accompanied by the infiltration of large, activated macrophages, epithelioid macrophages, and multinucleated giant cells, leading into terminal mineralization and complete loss of muscle mass. Metabolic precursors of nucleotide synthesis were significantly upregulated in the MP/HBOC group.

These precursors appear to be implicated in a strong regenerative response elicited by effective oxygenation of skeletal muscle, which also has a positive impact in energy utilization and ROS scavengers. The MP/HBOC also promoted effective *ex vivo* oxygenation and shifted skeletal muscle metabolic profile from β-oxidation towards Ω-oxidation during VCA preservation when compared to the prolonged anoxia under hypothermic conditions induced by CSP. This can be interpreted as a sign of mitochondrial dys-

function experienced by the CSP group. In fact, Ω-oxidation is linked to balanced redox state and less oxidative damage during stressful conditions induced by these experiments. In contrast, CSP appears to increase a reactive skeletal muscle β-oxidation pathway, which leads into oxidative damage and disintegration of cellular membranes when prolonged hypothermia, anoxia, and limited glucose supply is imposed. Contrary to the CSP group, MP/HBOC protects skeletal muscle against early graft myopathy. These complex metabolic features seen in the both the muscle and adipose tissue were extensively corroborated by the serial histological findings, revealing in a close analogy the same protective role exerted by effective ex-vivo oxygenation documented extensively in our previous liver experiments.

Final Conclusions

These pre-clinical experiments with large animal models (pigs) have shown the capabilities of the HBOC solution in promoting effective ex-vivo oxygenation in solid organ allografts (liver) and composed tissues (VCA). Both preservation protocols were conducted with MP devices that allowed continued pulsatile pressures and oxygenation over extended periods of time (e.g., 12-hours for the livers and 14 hours for the VCA).

The primary goal of the liver experiments was towards the characterization of a newly developed HBOC (OxyBridge). This initial and promising data showed the ability of this polymer to provide effective oxygenation to the liver tissue without inducing vasoconstriction and excessive ROS, despite the high pO2 levels (>500 mmHg) seen in the perfusate overtime. Moreover, the prompt impact on lactate clearance and the ability to sustain a stable pH overtime without the need for additional buffers was truly remarkable. OxyBridge ex-vivo allows the utilization of a wide range of temperatures, which is something unthinkable when standard RBCs components are utilized over extended periods of time.

The VCA experiments generated a massive amount of information in both the ex-vivo (during VCA perfusion with the MP/HBOC system) and in-vivo (after VCA transplantation) stages. These are the first experiments in VCA comparing the MP/HBOC system with cold static preservation (CSP) as the current standard of care for clinical applications. The histological findings are staggering, revealing clear full thickness necrosis of the allografts after the 14-hour preservation protocol. However, the same preservation time with the MP/HBOC system showed complete allograft viability over a 7-day period after the initial transplant, which is a remarkable finding when all the biproducts of the ischemia-reperfusion cascade were analyzed extensively with metabolomics and inflammatory markers.

References

1. Bing R. The perfusion of whole organs in the lindbergh apparatus with fluids containing hemocyanin as respiratory pigment. Science. 1938;87(2268):554–5.
2. Knowlton FP, Starling EH. The influence of variations in temperature and blood-pressure on the performance of the isolated mammalian heart. J Physiol. 1912;44(3):206–19.
3. Bainbridge FA, Evans CL. The heart, lung, kidney preparation. J Physiol. 1914;48(4):278–86.
4. Carrel A. The culture of whole organs. J Exp Med. 1937;65(4):515–26.
5. Starzl TE, Brittain RS, Stonnington OG, Coppinger WR, Waddell WR. Renal transplantation in identical twins. Arch Surg. 1963;86:600–7.
6. Lillehei RC, Goott B, Miller FA. The physiological response of the small bowel of the dog to ischemia including prolonged in vitro preservation of the bowel with successful replacement and survival. Ann Surg. 1959;150:543–60.
7. Ackermann JR, Barnard CN. Successful storage of kidneys. Br J Surg. 1966;53(6):525–32.
8. Brettschneider L, Daloze PM, Huguet C, Porter KA, Groth CG, Kashiwagi N, et al. The use of combined preservation techniques for extended storage of orthotopic liver homografts. Surg Gynecol Obstet. 1968;126(2):263–74.
9. Belzer FO, Ashby BS, Dunphy JE. 24-hour and 72-hour preservation of canine kidneys. Lancet. 1967;2(7515):536–8.
10. Etiology of rising perfusion pressure in isolated organ perfusion. [Internet]. [cited 2021 Jul 19]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1387342/.
11. Belzer FO, Southard JH. Principles of solid-organ preservation by cold storage. Transplantation. 1988;45(4):673–6.
12. Pienaar BH, Lindell SL, Van Gulik T, Southard JH, Belzer FO. Seventy-two-hour preservation of the canine liver by machine perfusion. Transplantation. 1990;49(2):258–60.
13. Dutkowski P, Schönfeld S, Odermatt B, Heinrich T, Junginger T. Rat liver preservation by hypothermic oscillating liver perfusion compared to simple cold storage. Cryobiology. 1998;36(1):61–70.
14. Dutkowski P, de Rougemont O, Clavien P-A. Rescue of the cold preserved rat liver by hypothermic oxygenated machine perfusion – PubMed. 2008;8(5):917–24.
15. Dutkowski P, Schlegel A, de Oliveira M, Müllhaupt B, Neff F, Clavien P-A. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol. 2014 Apr;60(4):765–72.
16. Neuhaus P, Blumhardt G. Extracorporeal liver perfusion: applications of an improved model for experimental studies of the liver. Int J Artif Organs. 1993;16(10):729–39.
17. Brockmann J, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D, et al. Normothermic perfusion: a new paradigm for organ preservation. Ann Surg. 2009;250(1):1–6.
18. Guarrera JV, Henry SD, Samstein B, Reznik E, Musat C, Lukose TI, et al. Hypothermic machine preservation facilitates successful transplantation of "orphan" extended criteria donor livers. Am J Transplant. 2015;15(1):161–9.
19. The 24-hour normothermic machine perfusion of discarded human liver grafts – PubMed [Internet]. [cited 2021 Jul 19]. Available from: https://pubmed.ncbi.nlm.nih.gov/27809409/.
20. Fontes P, Lopez R, van der Plaats A, Vodovotz Y, Minervini M, Scott V, et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. Am J Transplant. 2015;15(2):381–94.
21. Karangwa SA, Dutkowski P, Fontes P, Friend PJ, Guarrera JV, Markmann JF, et al. Machine perfusion of donor livers for transplantation: a proposal for standardized nomenclature and reporting guidelines. Am J Transplant. 2016;16(10):2932–42.

22. Curtis E, Hsu LL, Noguchi AC, Geary L, Shiva S. Oxygen regulates tissue nitrite metabolism. Antioxid Redox Signal. 2012;17(7):951–61.
23. MacArthur PH, Shiva S, Gladwin MT. Measurement of circulating nitrite and S-nitrosothiols by reductive chemiluminescence. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;851(1–2):93–105.
24. Pelletier MM, Kleinbongard P, Ringwood L, Hito R, Hunter CJ, Schechter AN, et al. The measurement of blood and plasma nitrite by chemiluminescence: pitfalls and solutions. Free Radic Biol Med. 2006;41(4):541–8.
25. Dutkowski P, de Rougemont O, Clavien P-A. Machine perfusion for "marginal" liver grafts. Am J Transplant. 2008;8(5):917–24.
26. Kim JS, He L, Qian T, Lemasters JJ. Role of the mitochondrial permeability transition in apoptotic and necrotic death after ischemia/reperfusion injury to hepatocytes. Curr Mol Med. 2003;3(6):527–35.
27. Petruzzo P, Dubernard JM. The international registry on hand and composite tissue allotransplantation. Clin Transpl. 2011:247–53.
28. Diaz-Siso JR, Bueno EM, Sisk GC, Marty FM, Pomahac B, Tullius SG. Vascularized composite tissue allotransplantation--state of the art. Clin Transpl. 2013;27(3):330–7.
29. Wall SP, Plunkett C, Caplan A. A potential solution to the shortage of solid organs for transplantation. JAMA. 2015;313(23):2321.
30. Cendales LC, Rahmel A, Pruett TL. Allocation of vascularized composite allografts: what is it? Transplantation. 2012;93(11):1086–7.
31. Chung KC, Oda T, Saddawi-Konefka D, Shauver MJ. An economic analysis of hand transplantation in the United States. Plast Reconstr Surg. 2010;125(2):589–98.
32. Jensen SE, Butt Z, Bill A, Baker T, Abecassis MM, Heinemann AW, et al. Quality of life considerations in upper limb transplantation: review and future directions. J Hand Surg Am. 2012;37(10):2126–35.
33. Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg. 2002;10(6):620–30.
34. Castillero E, Akashi H, Wang C, Najjar M, Ji R, Kennel PJ, et al. Cardiac myostatin upregulation occurs immediately after myocardial ischemia and is involved in skeletal muscle activation of atrophy. Biochem Biophys Res Commun. 2015;457(1):106–11.
35. Whitaker IS, Duggan EM, Alloway RR, Brown C, McGuire S, Woodle ES, et al. Composite tissue allotransplantation: a review of relevant immunological issues for plastic surgeons. J Plast Reconstr Aesthet Surg. 2008;61(5):481–92.
36. Lanzetta M, Petruzzo P, Vitale G, Lucchina S, Owen ER, Dubernard JM, et al. Human hand transplantation: what have we learned? Transplant Proc. 2004;36(3):664–8.
37. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. J Pathol. 2000;190(3):255–66.
38. Sadowsky D, Zamora R, Barclay D, Yin J, Fontes P, Vodovotz Y. Machine perfusion of porcine livers with oxygen-carrying solution results in reprogramming of dynamic inflammation networks. Front Pharmacol. 2016;7:413.
39. Metabolomics: from small molecules to big ideas | Nature Methods [Internet]. [cited 2021 Aug 16]. Available from: https://www.nature.com/articles/nmeth0211-117.
40. Jonsson P, Bruce SJ, Moritz T, Trygg J, Sjöström M, Plumb R, et al. Extraction, interpretation and validation of information for comparing samples in metabolic LC/MS data sets. Analyst. 2005;130(5):701–7.
41. Sadowsky D, Nieman G, Barclay D, Mi Q, Zamora R, Constantine G, et al. Impact of chemically-modified tetracycline 3 on intertwined physiological, biochemical, and inflammatory networks in porcine sepsis/ARDS. Int J Burns Trauma. 2015;5(1):22–35.
42. Vodovotz Y, An G. Systems biology and inflammation. Methods Mol Biol. 2010;662:181–201.
43. Dragu A, Birkholz T, Kleinmann JA, Schnürer S, Münch F, Cesnjevar R, et al. Extracorporeal perfusion of free muscle flaps in a porcine model using a miniaturized perfusion system. Arch Orthop Trauma Surg. 2011;131(6):849–55.
44. Müller S, Constantinescu MA, Kiermeir DM, Gajanayake T, Bongoni AK, Vollbach FH, et al. Ischemia/reperfusion injury of porcine limbs after extracorporeal perfusion. J Surg Res 2013;181(1):170–182.
45. Kamat P, Juon B, Jossen B, Gajanayake T, Rieben R, Vögelin E. Assessment of endothelium and inflammatory response at the onset of reperfusion injury in hand surgery. J Inflamm. 2012;9(1):18.
46. Almahmoud K, Abboud A, Namas RA, Zamora R, Sperry J, Peitzman AB, et al. Computational evidence for an early, amplified systemic inflammation program in polytrauma patients with severe extremity injuries. PLoS One. 2019;14(6):e0217577.

Resuscitation of Traumatic Hemorrhagic Shock

40

Sarayu Subramanian and Martin A. Schreiber

Introduction

Trauma is the leading cause of death in the 1–45 age group and is the fourth leading cause of death in all age groups globally. Exsanguination and traumatic brain injury (TBI) are the major contributors to mortality in trauma with exsanguination accounting for 60,000 deaths annually in the United States and 1.5 million deaths worldwide [1]. Death due to exsanguination occurs early after injury with 50% of deaths occurring in the prehospital setting or the first 6 hours of hospitalization. This is largely preventable and resuscitation practices play a major role in achieving hemorrhage control.

A multitude of factors contribute to bleeding and trauma-induced coagulopathy (TIC) is a global failure of the coagulation cascade. Coagulopathy is seen in 25% of severely injured patients and has been associated with a mortality rate of 35–40% [2]. TIC is initiated by an endogenous entity known as acute traumatic coagulopathy (ATC) and it is further perpetuated by the 'lethal triad' (Fig. 40.1). It is vital to tailor resuscitation practices to achieve hemostasis breaking the vicious cycle of coagulopathy.

Pathophysiology of Coagulopathy in Trauma

ATC is seen within minutes of trauma and it is unrelated to resuscitation practices [3]. ATC occurs in approximately one-third of severely injured patients and correlates with a higher requirement of blood product transfusion and a four-fold increase in mortality [3, 4]. Severe tissue injury, tissue hypoperfusion and endothelial disruption result in increased thrombin-thrombomodulin generation which activates Protein C [3, 5]. Activated protein C (APC) produces an anti-coagulant effect through (i) inhibition of factor V and VIII and (ii) fibrinolysis through inhibition of plasminogen activator inhibitor (PAI). Protein C activation has been corre-

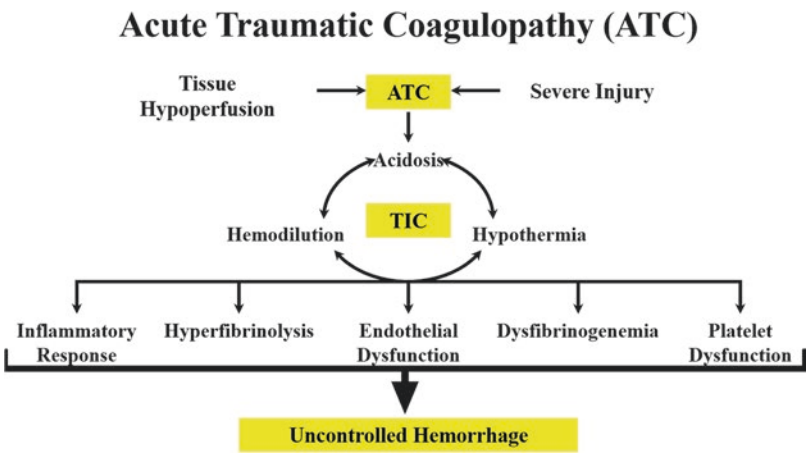


Fig. 40.1 Pathophysiology of coagulopathy in trauma

S. Subramanian · M. A. Schreiber (✉)
Department of Surgery, Division of Trauma and Acute Care Surgery, Oregon Health & Science University, Portland, OR, USA
e-mail: subramsa@ohsu.edu; schreibm@ohsu.edu

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_40

lated with decreased survival, increased transfusion requirements and critical care length of stay [3, 6]. Selective Protein C inhibition in mice models has been demonstrated to prevent early coagulopathy [7].

ATC causes a functional decline in clot generation and clot strength, typically measured by thrombelastometry (clot amplitude at 5 min < 35 mm) with minimal changes in conventional clotting assays [5]. Increased thrombin generation with peaks in thrombin concentration have been reported in the early phases of trauma [8, 9] which may explain the preserved clotting parameters.

Coagulopathy is further propagated by the lethal triad of hypothermia, acidosis and hemodilution exacerbated by suboptimal resuscitation practices. Severe hypothermia (<32 °C) decreases platelet activity and the synthesis of coagulation factors and fibrinogen. Metabolic acidosis in shock is due to anaerobic metabolism and lactic acid generation and it is worsened by resuscitation with crystalloids. Clotting factors exhibit suboptimal activity in an acidotic mileu and admission lactate levels have been correlated with increased mortality [10]. Additionally, anaerobic metabolism is less exothermic contributing to hypothermia. Hemodilution worsens coagulopathy by lowering the concentrations of cellular elements and clotting factors. Crystalloids although inexpensive and widely used, contain high concentrations of chloride leading to hyperchloremic metabolic acidosis.

The components of ATC and TIC perturb the hemostatic mechanism resulting in uncontrolled hemorrhage secondary to (i) endothelial dysfunction (ii) hyperfibrinolysis and dysfibrinogenemia (iii) platelet dysfunction and (iv) inflammatory response.

Traumatic hemorrhage and shock disrupt the glycocalyx due to tissue trauma, hypoperfusion, inflammation and catecholamine surge. Endothelial glycocalyx disruption results in heparan sulphate release, APC activation and fibrinolysis due to production of tissue plasminogen activator (tPA). tPA release is further enhanced by epinephrine and vasopressin [11].

Fibrinogen is the earliest clotting component to decline in trauma and early fibrinogen supplementation has been correlated with improved outcomes. Although the exact mechanism of fibrinogen depletion is not clearly understood, decreased production and hyperfibrinolysis have been proposed as underlying causes. Hypothermia and acidosis lower fibrinogen production, which is further exacerbated by hemodilution. The pro-coagulant properties of thrombin in fibrin generation is dampened with the activation of Protein C by the thrombin-thrombomodulin complex. In addition, decreased production of the thrombin activatable fibrinolysis inhibitor (TAFI) [12] shifts the dynamics to a hypocoagulable state.

Platelet dysfunction and dilutional thrombocytopenia secondary to platelet-excluding resuscitation practices contribute to platelet abnormalities. Platelet dysfunction despite normal counts has been reported in 86% of trauma patients correlating with increased in-hospital and 24-hour mortality rates [13]. "Exhausted platelet syndrome" has been described by Wolhauer et al. as a phenomenon of initial platelet hyperactivation due to widespread release of ADP by endothelial cells after which platelets become unresponsive. Impairment of the ADP pathway and proteins released from lysed platelets increase the sensitivity to tPA, enhancing fibrinolysis.

Trauma also results in activation of the inflammatory cascade. In the early phase of injury and tissue damage, endogenous triggers such as damage associated molecular patterns (DAMPs) or alarmins are released by the activated immune cells. DAMPs activate complement and inflammatory cells resulting in systemic inflammatory response syndrome (SIRS). This non-specific activation exhausts the body's immune response increasing susceptibility to infections via Pathogen-Associated Molecular Patterns (PAMPs). Activated platelets that are vital to clot formation also trigger the release of inflammatory cytokines. In contrast, APC has anti-inflammatory and cytoprotective effects, and its excessive activation and depletion results in loss of this protective effect. In vitro studies have suggested the cytoprotective role of APC in maintaining the pulmonary capillary endothelial barrier function and increased rates of pneumonia have been correlated with decreased APC levels [14, 15].

Resuscitation Practices in Trauma

Damage Control Resuscitation (DCR)

DCR evolved as an extension of damage control surgery and emerged as a strategic approach for bleeding trauma patients in extremis. The core concepts of DCR are to correct coagulopathy, attain hemostasis, and restore tissue perfusion to limit hypoxia [16]. Originating from military experience, DCR is also the standard of care now for civilian resuscitation practices. DCR comprehensively addresses all components of the lethal triad, and it is initiated after the rapid initial assessment of the patient in the emergency room and is continued through the operating room and ICU.

The components of DCR include: (i) hypotensive resuscitation to mitigate re-bleed (ii) appropriate hemostatic resuscitation (iii) early recognition of massive transfusion requirement (iv) prevention of acidosis and hypothermia (v) empirical use of hemostatic adjuncts to limit blood loss (vi) immediate arrest of hemorrhage.

Permissive Hypotension

Early and aggressive fluid administration to restore the circulating volume have been the cornerstones of traditional resuscitation in severe trauma and shock. Permissive hypotension is a restrictive approach for patients with severe injuries where minimal resuscitation to maintain tissue perfusion is maintained until active bleeding is controlled. This approach lowers the intravascular volume and mean arterial pressure (MAP), preserves local vasoconstriction, decreases the risk of clot dislodgement and hypothermia and avoids hemodilution thus decreasing the risk of active blood loss and re-bleeding [17]. However, there is a risk of tissue hypoperfusion and it is not indicated in patients with TBI or spinal cord injury.

Excessive infusion of saline-based fluids is associated with acidosis, coagulopathy, cardiac dysfunction, ACS, inflammation, ARDS, MODS, and increased mortality [18]. Balogh et al. correlated supranormal resuscitation with abdominal compartment syndrome even in the absence of abdominal injuries [19]. In a landmark study, Bickell et al. reported improved survival in penetrating torso patients whose fluid resuscitation was delayed until hemorrhage control was achieved. A follow-up prospective trial demonstrated survival benefits in a combined cohort of patients with blunt and penetrating trauma [20].

Schreiber et al. demonstrated the safety and feasibility of controlled resuscitation both in the pre-hospital and hospital settings in hypotensive trauma patients. In addition, controlled resuscitation correlated with early blood product transfusion, decreased volumes of crystalloid infusion and decreased 24-hour mortality in patients with blunt trauma [18]. A recent meta-analysis correlated permissive hypotension with improved in-hospital and 30-day mortality, decreased blood loss and fewer blood product requirements (pooled OR 0.7, 95% CI 0.53–0.92) [21].

Appropriate Hemostatic Resuscitation

The goal of resuscitation is to restore hemostasis by blood product transfusion that closely mimics the functionality of whole blood while limiting crystalloids to avoid hemodilution. The order of preference for fluid administration should be (i) whole blood (ii) 1:1:1 component ratio (plasma:RBC:platelet) (iii) plasma: RBC 1:1 (iv) plasma with or without RBC and (v) RBC alone.

Whole Blood

Whole blood (WB) transfusion is a balanced resuscitation that restores the cellular and coagulation components in physiological ratios. WB use offers the advantages of (i) transfusion of younger RBCs (ii) correction of coagulopathy because all components are present (iii) simplification of resuscitation logistics and (iv) decreased preservative use [22–24].

Transfusion practices evolved during World War II with the advent of component separation techniques and blood banking shifting the resuscitation practice from WB towards components which continued in the war in Vietnam [25]. Fractionation of blood into components allows for safe and targeted resuscitation with effective resource utilization. However, component resuscitation carries the risk of hemodilution. For instance, 180 ml of additives are required for the processing of a 500 ml donation into leukoreduced components. In addition, hemodilution due to mixing of components and addition of anti-coagulant and additive solution results in (i) drop in plasma coagulation factors to ~60% and hematocrit to less than 30%(ii) dilution of platelets to 80×10^9/L with only two thirds of platelets remaining viable and (iii) a loss of 15 ml of RBCs [26]. Hence, component resuscitation specifically in the setting of MT can worsen coagulopathy.

WB is typically administered as either fresh whole blood (FWB) in combat scenarios or as liquid cold stored WB that is stored at 1–6 °C. Type-specific fresh whole blood (FWB) can be stored at 22 ° C for up to 8 hours and can be stored up to 48 hours at 1–6 °C. The logistic constraints of blood component storage and resuscitation, specifically platelets led to the use of FWB in deployed environments. The WB that is collected on site in these settings is obtained from a select donor population and undergoes rapid testing that is 85% sensitive for transfusion-transmitted diseases. An aliquot of this blood is sent for formal testing. Type-specific FWB availability can be challenging due to a limited pool of donors with ABO compatibility. Type O FWB has been shown to be safe when used as a universal donor [22]. Recent studies have associated FWB with decreased mortality in the military setting [27, 28]. US military guidelines recommend FWB for patients requiring MT when component therapy is unavailable. Nessen et al. reported the overall safety and survival benefit of FWB used in addition to components (RBC and FFP) when compared to components alone even in the setting of MT requirement in combat settings.

Cold stored WB with low titres of anti-A and anti B is used in civilian setting because FWB is not approved for use due to inherent transfusion-transmitted disease potential. Although there are no accepted guidelines to define low titres, IgM <200 is the commonly used cut-off by most institutions. LTOWB can be stored for 21 days with the addition of minimal volume of CPD/CP2D solution, and the addition of CPDA-1 solution extends the shelf life to 35 days. Additionally, LTOWB that is unused within its shelf life can be fractionated into pRBC thus avoiding blood wastage. The

current AABB guidelines requiring ABO compatibility only for RBCs, has made LTOWB resuscitation feasible in the early hospital and prehospital course where the recipient ABO status is often unknown.

Data on the use of WB in civilian trauma is limited, and most studies are retrospective comparing combined use of WB plus components to components alone. Retrospective studies from civilian settings have shown WB transfusion to be safe with evidence of decreased component use. Prospective studies from University of Pittsburgh and the University of Texas Health Center showed improved plasma/RBC ratio but no survival benefits with LTOWB when used in small volumes (<6 units) except in a small subset of patients with traumatic brain injury [29–31]. The follow-up trial from University of Pittsburgh by Seheul et al. comparing LTOWB with conventional component therapy showed no significant differences in outcomes or transfusion requirement, although the time to normalization of lactate levels was shorter in the LTOWB group [32]. Additionally, in a recent systematic review of retrospective studies by Malkin et al., WB was found to be safe with no survival benefit or reduced transfusion requirement [33]. A study by Shea et al. from Barnes in St. Louis did show reduced component utilization and improved survival in patients who received LTOWB in addition to components compared to patients who received components alone [34].

In the setting of massive transfusion, Gallaher et al. demonstrated that LTOWB in addition to component therapy was found to be safe and feasible with no differences in 24-hour and 30-day mortality when compared with component resuscitation alone. Furthermore, this study showed that LTOWB transfusion also better approximates the 1:1:1 ratio of RBC:platelets:plasma [35], and this ratio has been associated with improved hemostasis and fewer deaths from exsanguination in the PROPPR trial [23]. Additionally, a recent case report from the US with MT of WB (38 units) along with components was found to be safe [36].

WB is often leukoreduced to lower the risk of pathogen transmission, transfusion reactions and alloimmunization to HLA due to WBCs. The initial leukoreduction also filtered platelets, which was later modified with platelet sparing filters. Studies have shown that leukoreduction with a platelet sparing filter still causes an early reduction in platelet count and function [37, 38], although this effect is not persistent and does not have well established clinical implications. Additionally, effects of leukoreduction on platelet number and function are attenuated on cold stored WB, with improved platelet function observed when stored at 4 °C [39–41].

WB transfusion even with LTOWB raises the concern of Rh immunisation in child-bearing women, which can be addressed by using Rh negative LTOWB, albeit a scarce resource. The benefit of LTOWB even with Rh positive WB in exsanguination outweighs the low future risk (0.3%) of alloimmunization.

Blood Product Ratio

Considerable variations in transfusion practices prevail across various level 1 trauma centres, although high component ratios closely resemble the functionality of whole blood. The PROMMTT study demonstrated the time varying nature of component resuscitation in trauma. 1:1 or 1:2 (plasma: RBC or platelets: RBC) were the most common component ratios transfused, with only 72% of patients receiving platelets and the median time to death due to exsanguination was 3 hours [42]. Higher blood product ratios correlated with lower early in-hospital mortality within the first 6-hours, following which this protective effect diminished. This trend is explained by the dynamic course of events in traumatic injuries, with non-hemorrhagic complications playing a bigger role in mortality after the first 24-hours of admission. The multisite phase 3 PROPPR trial compared the safety and efficacy of early 1:1:1 (n = 338) with 1:1:2 (n = 342) component resuscitation in patients at risk for severe hemorrhage who were predicted to require MT (>10 U RBCs within 24 hours). Mortality due to exsanguination was significantly lower in the 1:1:1 group both within the first 24 hours and 30-days post-injury; and a higher percentage of patients attained hemostasis. No differences in 24-hour mortality, 30-day mortality or safety profile were seen between the groups.

Plasma

Resuscitation with plasma has been demonstrated to improve survival and reverse the coagulopathy in trauma even in the absence of PRBC transfusions [43, 44]. The beneficial effects of plasma in trauma are not limited to replenishing clotting factors alone. Plasma also helps in volume expansion, provides fibrinogen (2 g/L or 0.6 g/300 ml) and improves acidosis by acting as a buffer [45]. Furthermore, plasma has been demonstrated to stabilize systemic vascular architecture by modulating the endotheliopathy of trauma. Plasma also reduces inflammation, edema and vascular permeability by repair of the glycocalyx and tight junctions [46]. Plasma has been shown to reduce inflammation in animal models and it also lowers the risk of hypercoagulability by regulating thrombin generation [47–49].

Plasma is predominantly used as fresh frozen plasma (FFP) in the US and is stored frozen at −20 °C. FFP is either derived from whole blood units or from plasma collected by apheresis in a citrated solution and frozen within 8 hours

(FFP). Type AB plasma is the universal donor but type A or type specific plasma can also be used as only 4% of donors are type AB. FFP has a shelf life of 365 days and is thawed prior to transfusion. Thawing of plasma takes around 20–30 minutes and thawed plasma can be stored at 1–6 °C for up to 5 days. The recent PAMPer trial demonstrated significantly improved 30-day mortality associated with prehospital thawed plasma use when compared to standard resuscitation (23.2% vs. 33%, difference − 9.8%, p = 0.03) [50]. The COMBAT trial found no survival benefit with early plasma but transport times were very short [51]. Transfusion requirements for trauma patients are often unpredictable and it can be challenging to find a balance between thawing adequate plasma to avoid delay in resuscitation and avoiding wastage of valuable resources. Thawed plasma stored at 4 ° C has the advantage of being immediately available for emergent scenarios [46, 52].

Plasma is associated with risks of transfusion associated lung injury (TRALI), ARDS, hypocalcelima, blood-borne disease transmission and a potential for fluid overload which can be limiting in the setting of cardiac/renal or liver failure. FP24 is plasma that is stored within 8–24 hours of donation and its clotting factor levels are comparable to that of FFP [53]. Longer time from donation allows for adequate time to test HLA antigen thus lowering the risk of TRALI [54]. Most blood banks only use plasma from males to also reduce the risk of TRALI.

Liquid plasma (LQP) is an alternative form of plasma that is never frozen, and it is stored at 2–6 ° C for up to 26 days. LQP has the advantages of (i) ease of availability (ii)superior clotting factor profiles when compared to fresh frozen plasma and sustained hemostatic benefits throughout storage [55].

Massive Transfusion Protocol

3% of civilian trauma patients and 8% of military injuries are reported to require massive transfusion (MT). Such patients have a 40–60% mortality rate and utilize 70% of the overall blood transfused in trauma centers [56]. MT calls for an unprecedented requirement of massive volumes of blood products that must be coordinated and rapidly delivered with a multidisciplinary team involving the trauma service, emergency department, blood bank, anaesthesia, and the operating room. The development of a massive transfusion protocol (MTP) has been associated with better patient outcomes with reduced mortality and effective resource utilization. MTP allows for empirical treatment in patients with severe hemorrhage where resuscitation cannot be put on hold awaiting laboratory parameters.

The definition for massive transfusion (MT) continues to evolve and the current widely accepted definition is transfusion of more than 10 units of PRBC over a 24-hour period. The historical definition with a 24-hour time frame is often futile in managing a patient with ongoing hemorrhage and the intensity of resuscitation is not often taken into account. Additionally, critically ill patients succumb to exsanguination before satisfying the transfusion criteria. These shortcomings are addressed by the critical administration threshold (CAT) which defines massive transfusion as receipt of at least 3 units of blood in a 60 minute time period [57].

Modern MTP protocols are formulated with a balanced component resuscitation approach. Death due to exsanguination typically occurs in the first 6 hours and most institutions require a longer survival time to implement higher component ratios. Early prediction of MT although challenging is critical for improving outcomes. The Assessment of Blood Consumption (ABC) score is a widely accepted scoring system that helps in rapid and easy identification of such patients in the trauma bay. It is based on four equally weighted parameters (i) pulse >120/min (ii) SBP <90 mm Hg (iii) positive FAST and (iv) penetrating torso trauma and a score ≥ 2 warrants MTP activation (PPV 50–55%, NPV 95%).

MTP activation requires significant preplanning to rapidly deliver preset boxes of blood components in predetermined ratios and sequences until hemorrhage slows after which resuscitation can be guided by laboratory parameters. MTP at a center should be a well written-easily accessible document, with initial training and subsequent drills to maintain competency. Cotton et al. showed MTP with early protocol activation with pre-defined blood product ratio was an independent predictor of survival and reduced blood product wastage [30].

Once the MTP is activated, the blood bank delivers plasma and RBCs in a 1:1 ratio. One apheresis unit of platelets is required for each 6 units of RBCs and plasma to maintain a 1:1:1 ratio. When the recipient blood type is unknown, transfusion with O Rh + RBCs is appropriate for all patients except for females of childbearing age who require Rh- blood. Type AB or type A plasma is utilized until type specific product can be delivered. Further boxes of 6:6:1 components are prepared as mandated by the hemostatic requirements of the bleeding patient. For actively bleeding patients identified within 3 hours of injury, TXA is administered as 2 g bolus. Figure 40.2 describes a typical MTP activation at Oregon Health & Science University.

Baseline and serial monitoring of laboratory parameters to assess coagulopathy (INR, aPTT, fibrinogen, CBC), arterial blood gas, ionized calcium and point-of care testing (ROTEM/TEG) is necessary. Thrombelastometry should be ordered at the initiation of MTP and every 30–60 minutes in case adjunctive blood products are required to achieve normal coagulation.

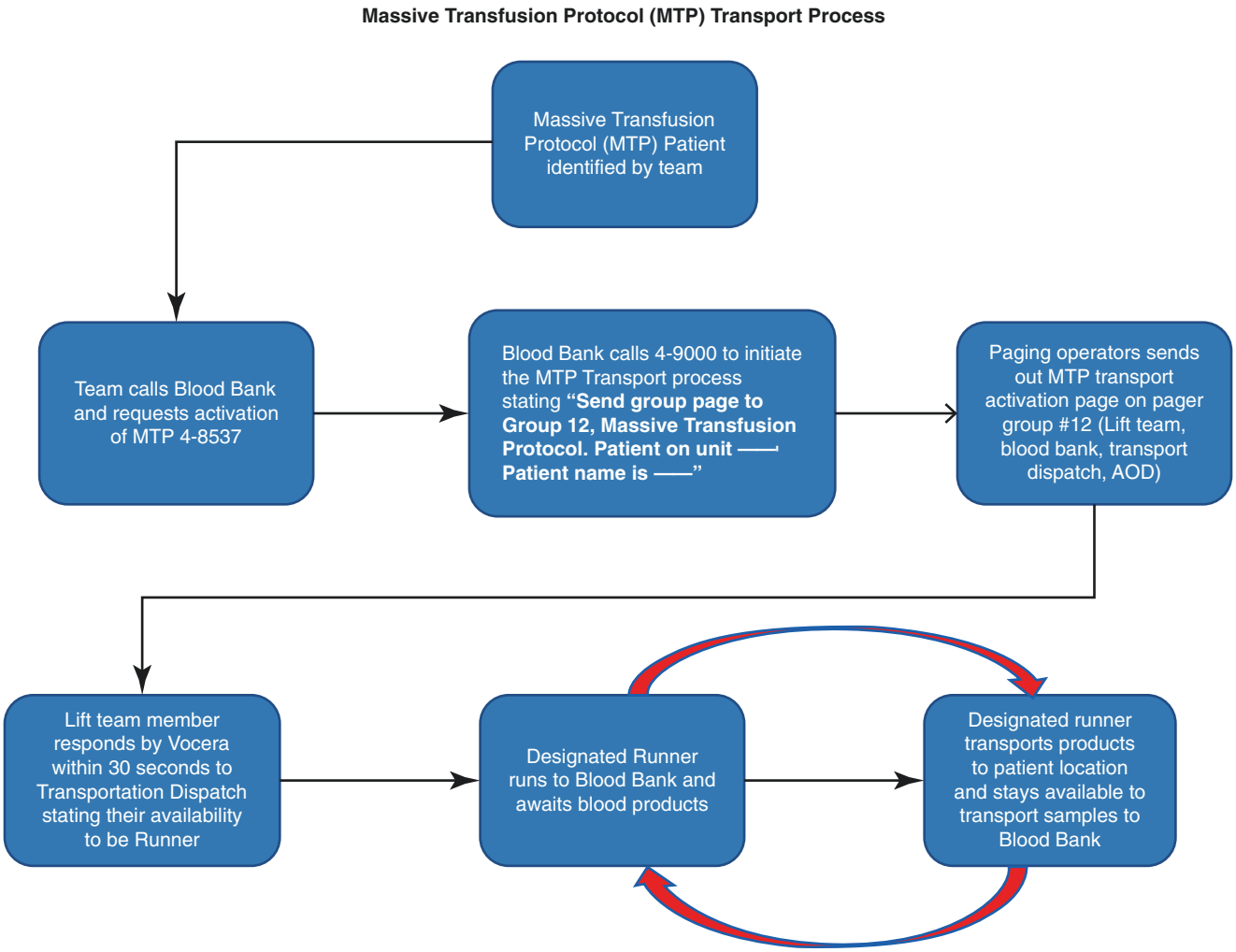


Fig. 40.2 Massive transfusion protocol at Oregon Health & Science University

MT carries risks related to storage and transfusion of large volumes of blood products. Hypocalcemia secondary to calcium chelation by citrated blood products is potentiated with hemorrhagic shock, rapid blood product resuscitation and decreased hepatic clearance. A common practice is to give calcium gluconate after 4 units of blood product administration and to monitor ionized calcium levels every 30–60 minutes for the duration of the MTP. Other risks include hyperkalemia, acidosis, transfusion associated cardiac overload (TACO) and TRALI.

Adjuncts

Tranexamic Acid

Tranexamic acid (TXA) is an anti-fibrinolytic agent and has long been used for reducing blood loss in the perioperative setting in elective surgeries and for short-term prevention of bleeding in patients with hemophilia. TXA inhibits the kinase dependent activation of plasminogen to plasmin thereby preventing fibrinolysis. In TBI, this kinase-blocking action of TXA has been shown to maintain vascular endothelial integrity by attenuating glycocalyx breakdown and thus mitigating further coagulopathy [58].

Henry et al. in a Cochrane database review of 20,781 elective surgery patients demonstrated that TXA use reduced blood loss and transfusion requirements (RR 0.61, 95% CI 0.54–0.70), with no impact on mortality, thromboembolic or cardiovascular events [59]. The CRASH-2 (Clinical Randomisation of an Antifibrinolytic in Significant Hemorrhage 2) trial assessed the role of TXA in 20,211 hypotensive adult trauma patients across 274 hospitals in 40 countries. Bolus infusion of 1 g of TXA over 10 minutes followed by another 1 g over 8 hours correlated with decreased all cause-mortality (16% vs. 14.5%, RR 0.91, 95% CI 0.85–0.97, p–0.0035) and the risk of death due to exsanguination (RR O.85, 95% CI 0.76–0.96, p = 0.0077) [60].

Post-hoc analysis of the CRASH-2 trial demonstrated that early TXA use within 1 hr. and within 3 hr. significantly lowered the risk of death due to bleeding by 2.4% and 1.6%, respectively. In contrast, delayed TXA administration increased the risk of death due to exsanguination possibly due to elevated PAI-1 levels at a later stage.

Kcentra

The four-factor prothrombin complex concentrate (PCC, proprietary name Kcentra) contains the vitamin K dependent coagulation factors (II, VII, IX, X), Protein C, Protein S, antithrombin III, heparin, protein Z and other unlisted proteins whose functions are not fully understood. PCC has a higher concentration of clotting factors when compared to FFP and does not require thawing. It is now increasingly used in trauma either alone or in conjunction with FFP.

PCC is available as a lyophilized powder, easily reconstituted and allows for rapid intravenous administration in small volumes. Thus, PCC has logistic benefits in austere, rural, and prehospital settings as an adjunct for blood products. Originally developed for the treatment of hemophilia, PCC use has evolved with two current FDA- approved indications: (i) reversal of warfarin-induced bleeding and (ii) for patients requiring major surgeries or invasive procedures who are on warfarin.

PCC use in conjunction with FFP and fibrinogen has been shown to have beneficial effects in trauma [61, 62]. A propensity analysis on a large American College of Surgeons (ACS) Trauma Quality Improvement Program dataset (TQIP) (FFP n = 243, FFP + PCC =243) showed use of PCC in conjunction with FFP was associated with significantly improved in-hospital mortality, decreased requirement of plasma and pRBC at 4 hours and 24 hours following admission. Additionally, there was a lower risk of AKI and ARDS possibly due to the lower volumes of FFP transfused. Kuckelman et al. demonstrated improved clotting time, coagulopathy and decreased lactic acidosis in a hemorrhagic swine model, while Jehan et al. showed decreased requirements of pRBC and FFP and earlier correction of INR when PCC was administered along with FFP in patients with severe hemorrhage [61, 62].

PCC has been shown to have favourable effects on the endotheliopathy of trauma similar to FFP in mice models [63]. In a follow up study on rat models, PCC did not show discernible benefits on the pulmonary vasculature, although there was a moderately increased clotting potential in shocked animals which suggests the possible independent effects of PCC in EOT and coagulopathy [64].

PCC has a theoretical risk of thromboembolic complications due to its higher concentration of clotting factors. Studies have shown divergent conclusions about the of thromboembolic risk of PCC and hence PCC use in trauma calls for a cautious approach with careful assessment of risk-benefit profile.

Cryoprecipitate

Cryoprecipitate (CP) is the current standard of care for fibrinogen supplementation in bleeding trauma patients with dysfibrinogenemia [65]. Cryoprecipitate (CP) consists of factor VIII and vWF,, fibrinogen, fibronectin, platelet microparticles and other plasma proteins that decrease fibrinolysis [66]. CP is derived from WB or through apharesis and is prepared by thawing FFP to 1–6 ° C and extracting the precipitate which is refrozen within an hour of thawing to −18 ° C. Crossmatching is not required due to the small volume of plasma transfused. It has a shelf-life of 12 months. Each unit contains at least 80 IU of factor VIII and 150 mg of fibrinogen and its fibrinogen content per unit of CP is widely variable (7–30 g/L). However, when used in the setting of hypofibrinogenemia, it allows for administration of smaller volumes when compared to FFP (100 ml CP vs. 1000 ml FFP) [67]. CP needs to be thawed prior to use which causes a delay in its administration and it must be given within 4–6 hours of thawing potentially leading to resource wastage.

The use of CP in resuscitating bleeding trauma patients is relatively uncommon and varies across trauma centres. There is paucity of data on its use as an adjunct to transfusion [68]. Additionally, CP is more commonly administered late in MTP and after hypofibrinogenemia has developed (fibrinogen levels <1 g/L). In a recent study, CP was shown to decrease endotheliopathy of trauma in vivo and invitro, similar to the effects of FFP [69]. A retrospective review of severely injured patients requiring MT in the ACS-TQIP database demonstrated a significant decrease in 24-hour mortality (24% vs. 31%, $p < 0.01$, OR 0.78) and in-hospital mortality (42% vs. 49%, $p < 0.01$, OR 0.79) for patients receiving CP as an adjunct to PRBC. No increase in in-hospital complication rates were noted with CP use [70]. The CRYOSTAT-1 trial demonstrated the feasibility of delivering CP within 90 minutes of admission (median time 60 min), with early administration correlating with a fibrinogen level of over 1.8 g/L [71]. The CRYOSTAT-2 trial ISRCTN 14998314) is currently underway to assess the role of early supplementation of high-dose fibrinogen in the form of CP in reducing mortality in patients requiring MT.

Fibrinogen Concentrate

Critically low concentrations of fibrinogen have been associated with massive bleeding, increased transfusion requirements, impaired hemostasis and poor clinical outcomes in severe trauma [72–75]. The current European guidelines recommend maintaining a fibrinogen level of 1.5–2 g/l in patients with significant hemorrhage and with an evidence of functional fibrinogen deficit on thromboelastometry [76]. The optimal timing, method and dose of fibrinogen remains debated. There is growing interest over the past decade in fibrinogen supplementation for patients with severe hemorrhage [77] and this can be achieved by the administration of (a) Fresh Frozen Plasma (FFP) (b) Cryoprecipitate (CP) and (c) Fibrinogen concentrate (FC). FC derived from human plasma, is virus inactivated by pasteurization and potentially free of antibodies thus conferring the advantages of decreased risk of blood borne pathogen transmission and adverse effects related to allogenic blood product use. The lyophilized form of FC is readily available, can be easily reconstituted and rapidly administered even in the pre-hospital setting with a shelf life of 5 years when stored at room temperature (25 ° C). FC allows for administration of higher concentration of fibrinogen with smaller volumes (20 g/l, 200 ml to administer 4 g fibrinogen) when compared to FFP (2 g/L) or CP (8–16 g/L).

In a retrospective analysis of 128 patients by Scochl et al., thromboelastometry-guided administration of FC in a dose of 2–4 gm in conjunction with PCC was demonstrated to have beneficial effects with an observed mortality that was lower than predicted based on TRISS methodology [78]. The recent FiiRST trial has demonstrated the early FC use in trauma is feasible and is associated with increased plasma fibrinogen levels in hemorrhage [79]. However, Hamada et al. in a recent study on 1027 patients with hemorrhagic shock from the French Trauma registry demonstrated no significant benefits with fibrinogen administration within 6 hours on 24-hour mortality [risk difference − 0.031 95% CI (−0.084–0.021)]. Hence, it is desirable to further evaluate the timing of FC administration on outcomes in trauma [80]. Although fibrinogen supplementation raises significant concerns about the risk of thromboembolic complications, data from prior studies have shown no evidence of such risk [81].

ROTEM and TEG Guided Resuscitation

Viscoelastic hemostatic assays (VHA) assess overall clot formation in whole blood providing numerical and graphical details about the kinetics of clot formation in a single assay. The VHAs capture the evolution of a clot as opposed to the cross-sectional details obtained from conventional coagulation tests (CCT). CCTs are not ideal in predicting TIC, do not accurately reflect fibrin formation and are time consuming. Hyperfibrinolysis is associated with higher mortality when identified by VHAs. TEG and ROTEM, the two commonly used VHAs provide a graphical presentation of clot formation with time on the x-axis and strength of the clot on the y-axis. VHAs have been proven to be effective in decreasing transfusion requirements, morbidity and health care costs [82]. A typical TEG and ROTEM tracing is described in Fig. 40.3a and Fig. 40.3b, respectively [83]. VHAs can depict specific coagulation abnormalities enabling goal-directed resuscitation and monitoring (Fig. 40.4 [84]) of post-injury coagulation status thus avoiding complications from overzealous resuscitation.

VHAs) should be ordered in patients receiving or requiring MT and a goal-directed resuscitation should be carried out based on an institutional algorithm. Schöchl et al. demonstrated a significant association between ROTEM use and decreased transfusion requirements of platelets and PRBC [78, 85]. The same group also predicted the need for MTP initiation based on FIBTEM values and the survival benefit from ROTEM-guided resuscitation when compared to predicted mortality from injury severity.

Summary

Resuscitation strategies for exsanguinating trauma patients have continued to evolve over the past two decades and appropriate hemostatic resuscitation has now emerged as a pivotal component of damage control resuscitation to reverse coagulopathy [41]. Whole blood administration when feasible and balanced component resuscitation along with the use of adjuncts while limiting aggressive crystalloid administration improves outcomes in traumatic hemorrhage.

Key Points

- Hemorrhage is a major cause of preventable mortality in trauma and multiple factors contribute to coagulopathy.
- Appropriate hemostatic resuscitation practices with a goal to closely resemble the functionality of blood is vital.
- Whole blood resuscitation is superior followed by balanced component resuscitation and early recognition of massive transfusion requirement is critical.
- Early plasma administration is beneficial.
- Resuscitation should be goal directed with appropriate use of adjuncts and guided by viscoelastic assays.

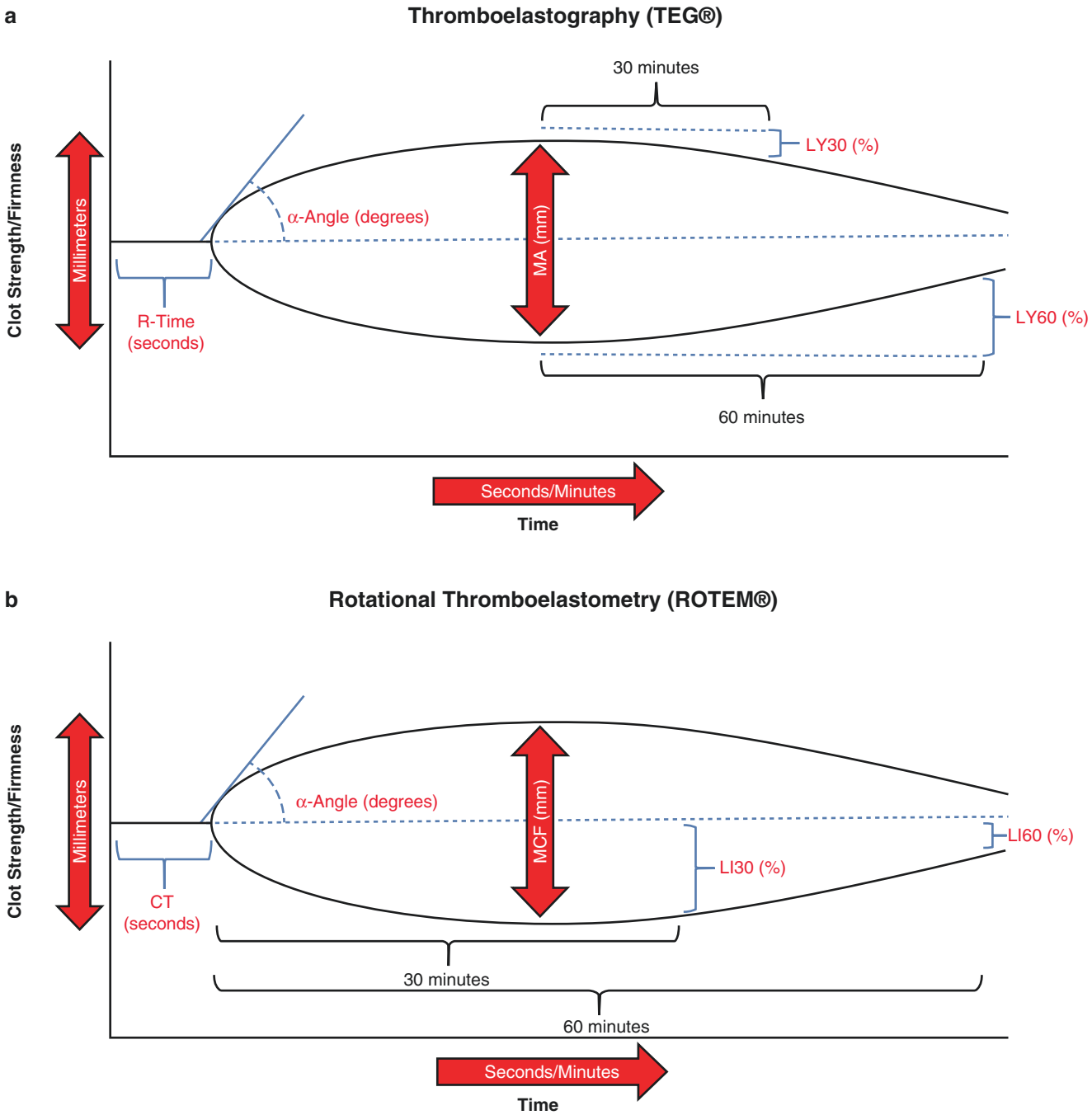


Fig. 40.3 (**a**) shows TEG tracing and (**b**) shows ROTEM tracing

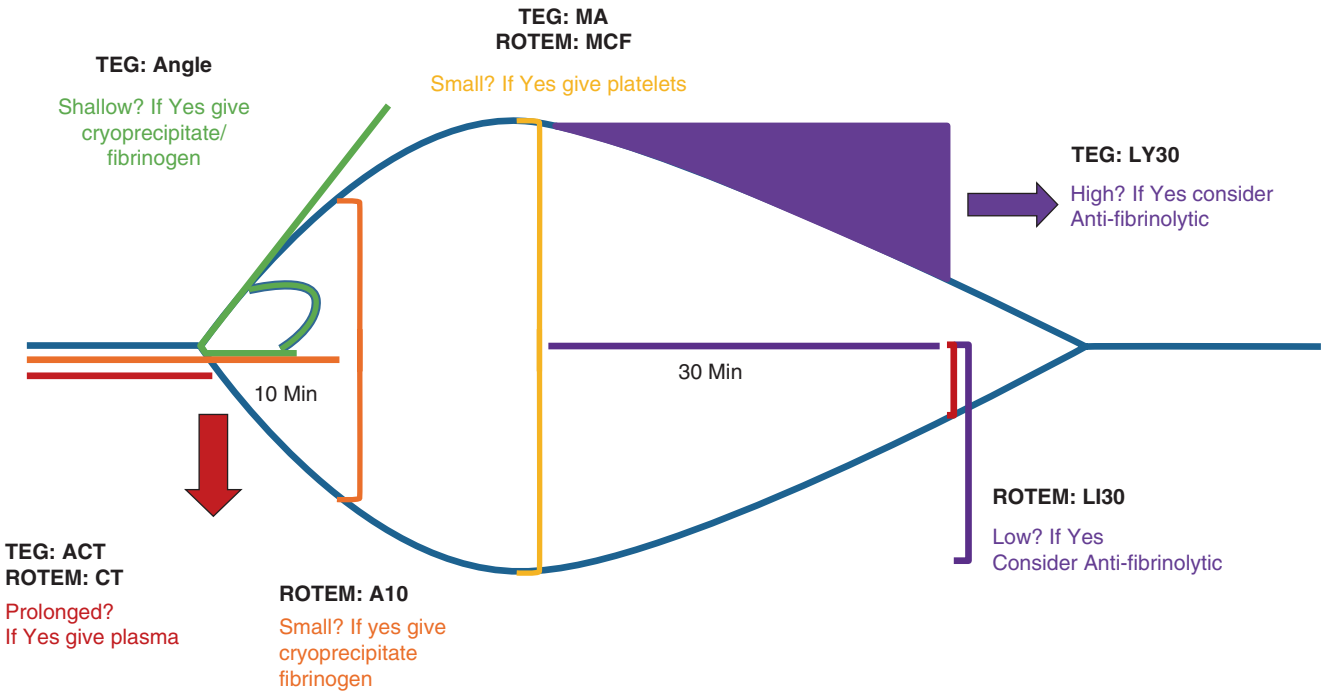


Fig. 40.4 TEG and ROTEM guided resuscitation

References

1. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012;380(9859):2095–128.
2. Kushimoto S, Kudo D, Kawazoe Y. Acute traumatic coagulopathy and trauma-induced coagulopathy: an overview. J Intensive Care. 2017;5(1):6.
3. Brohi K, Singh J, Heron M, Coats T. Acute traumatic coagulopathy. J Trauma. 2003;54(6):1127–30.
4. Maegele M, Lefering R, Yucel N, Tjardes T, Rixen D, Paffrath T, et al. Early coagulopathy in multiple injury: an analysis from the German Trauma Registry on 8724 patients. Injury. 2007;38(3):298–304.
5. Davenport R, Manson J, De'Ath H, Platton S, Coates A, Allard S, et al. Functional definition and characterisation of acute traumatic coagulopathy. Crit Care Med. 2011;39(12):2652–8.
6. Cohen MJ, Call M, Nelson M, Calfee CS, Esmon CT, Brohi K, et al. Critical role of activated protein C in early coagulopathy and later organ failure, infection and death in trauma patients. Ann Surg. 2012;255(2):379–85.
7. Chesebro BB, Rahn P, Carles M, Esmon CT, Xu J, Brohi K, et al. Increase in activated protein C mediates acute traumatic coagulopathy in mice. Shock. 2009;32(6):659–65.
8. Dunbar NM, Chandler WL. Thrombin generation in trauma patients. Transfusion. 2009;49(12):2652–60.
9. Matijevic N, Wang Y-WW, Wade CE, Holcomb JB, Cotton BA, Schreiber MA, et al. Cellular microparticle and thrombogram phenotypes in the Prospective Observational Multicenter Major Trauma Transfusion (PROMMTT) study: correlation with coagulopathy. Thromb Res. 2014;134(3):652–8.
10. Tremblay LN, Feliciano DV, Rozycki GS. Assessment of initial base deficit as a predictor of outcome: mechanism of injury does make a difference. Am Surg. 2002;68(8):689–93; discussion 693–4.
11. Cardenas JC, Matijevic N, Baer LA, Holcomb JB, Cotton BA, Wade CE. Elevated tissue plasminogen activator and reduced plasminogen activator inhibitor promote hyperfibrinolysis in trauma patients. Shock. 2014;41(6):514–21.
12. Bajzar L, Jain N, Wang P, Walker JB. Thrombin activatable fibrinolysis inhibitor: not just an inhibitor of fibrinolysis. Crit Care Med. 2004;32(5 Suppl):S320–4.
13. Wohlauer MV, Moore EE, Thomas S, Sauaia A, Evans E, Harr J, et al. Early platelet dysfunction: an unrecognized role in the acute coagulopathy of trauma. J Am Coll Surg. 2012;214(5):739–46.
14. Bouwens EAM, Stavenuiter F, Mosnier LO. Mechanisms of anticoagulant and cytoprotective actions of the protein C pathway. J Thromb Haemost. 2013;11(1):242–53.
15. Finigan JH, Dudek SM, Singleton PA, Chiang ET, Jacobson JR, Camp SM, et al. Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. J Biol Chem. 2005;280(17):17286–93.
16. Holcomb JB, Jenkins D, Rhee P, Johannigman J, Mahoney P, Mehta S, et al. Damage control resuscitation: directly addressing the early coagulopathy of trauma. J Trauma. 2007;62(2):307–10.
17. Morrison CA, Carrick MM, Norman MA, Scott BG, Welsh FJ, Tsai P, et al. Hypotensive resuscitation strategy reduces transfusion requirements and severe postoperative coagulopathy in trauma patients with hemorrhagic shock: preliminary results of a randomized controlled trial. J Trauma. 2011;70(3):652–63.
18. Schreiber MA, Meier EN, Tisherman SA, Kerby JD, Newgard CD, Brasel K, et al. A controlled resuscitation strategy is feasible and safe in hypotensive trauma patients: results of a prospective randomized pilot trial. J Trauma Acute Care Surg. 2015;78(4):687–95; discussion 695–7
19. Balogh Z, McKinley BA, Cocanour CS, Kozar RA, Valdivia A, Sailors RM, et al. Supranormal trauma resuscitation causes more cases of abdominal compartment syndrome. Arch Surg. 2003;138(6):637–42; discussion 642–3.

20. Bickell WH, Wall MJ, Pepe PE, Martin RR, Ginger VF, Allen MK, et al. Immediate versus delayed fluid resuscitation for hypotensive patients with penetrating torso injuries. N Engl J Med. 1994;331(17):1105–9.
21. Tran A, Yates J, Lau A, Lampron J, Matar M. Permissive hypotension versus conventional resuscitation strategies in adult trauma patients with hemorrhagic shock: a systematic review and meta-analysis of randomized controlled trials. J Trauma Acute Care Surg. 2018;84(5):802–8.
22. Yazer MH, Cap AP, Spinella PC. Raising the standards on whole blood. J Trauma Acute Care Surg. 2018;84(6S Suppl 1):S14–7.
23. Holcomb JB, Tilley BC, Baraniuk S, Fox EE, Wade CE, Podbielski JM, et al. Transfusion of plasma, platelets, and red blood cells in a 1:1:1 vs a 1:1:2 ratio and mortality in patients with severe trauma: the PROPPR randomized clinical trial. JAMA. 2015;313(5):471–82.
24. Davenport RA, Brohi K. Cause of trauma-induced coagulopathy. Curr Opin Anaesthesiol. 2016;29(2):212–9.
25. Hardaway RM. Wound shock: a history of its study and treatment by military surgeons. Mil Med. 2004;169(7):iv.
26. Armand R, Hess JR. Treating coagulopathy in trauma patients. Transfus Med Rev. 2003;17(3):223–31.
27. Nessen SC, Eastridge BJ, Cronk D, Craig RM, Berséus O, Ellison R, et al. Fresh whole blood use by forward surgical teams in Afghanistan is associated with improved survival compared to component therapy without platelets. Transfusion. 2013;53(Suppl 1):107S–13S.
28. Spinella PC, Perkins JG, Grathwohl KW, Beekley AC, Holcomb JB. Warm fresh whole blood is independently associated with improved survival for patients with combat-related traumatic injuries. J Trauma. 2009;66(4 Suppl):S69–76.
29. Yazer MH, Jackson B, Sperry JL, Alarcon L, Triulzi DJ, Murdock AD. Initial safety and feasibility of cold-stored uncrossmatched whole blood transfusion in civilian trauma patients. J Trauma Acute Care Surg. 2016;81(1):21–6.
30. Cotton BA, Podbielski J, Camp E, Welch T, del Junco D, Bai Y, et al. A randomized controlled pilot trial of modified whole blood versus component therapy in severely injured patients requiring large volume transfusions. Ann Surg. 2013;258(4):527–32; discussion 532–3
31. Seheult JN, Bahr M, Anto V, Alarcon LH, Corcos A, Sperry JL, et al. Safety profile of uncrossmatched, cold-stored, low-titer, group O+ whole blood in civilian trauma patients. Transfusion. 2018;58(10):2280–8.
32. Seheult JN, Anto V, Alarcon LH, Sperry JL, Triulzi DJ, Yazer MH. Clinical outcomes among low-titer group O whole blood recipients compared to recipients of conventional components in civilian trauma resuscitation. Transfusion. 2018;58(8):1838–45.
33. Malkin M, Nevo A, Brundage SI, Schreiber M. Effectiveness and safety of whole blood compared to balanced blood components in resuscitation of hemorrhaging trauma patients – a systematic review. Injury. 2021;52(2):182–8.
34. Shea SM, Staudt AM, Thomas KA, Schuerer D, Mielke JE, Folkerts D, et al. The use of low-titer group O whole blood is independently associated with improved survival compared to component therapy in adults with severe traumatic hemorrhage. Transfusion. 2020;60(Suppl 3):S2–9.
35. Gallaher JR, Dixon A, Cockcroft A, Grey M, Dewey E, Goodman A, et al. Large volume transfusion with whole blood is safe compared with component therapy. J Trauma Acute Care Surg. 2020;89(1):238–45.
36. Condron M, Scanlan M, Schreiber M. Massive transfusion of low-titer cold-stored O-positive whole blood in a civilian trauma setting. Transfusion. 2019;59(3):927–30.
37. Remy KE, Yazer MH, Saini A, Mehanovic-Varmaz A, Rogers SR, Cap AP, et al. Effects of platelet-sparing leukocyte reduction and agitation methods on in vitro measures of hemostatic function in cold-stored whole blood. J Trauma Acute Care Surg. 2018;84(6S Suppl 1):S104–14.
38. Pidcoke HF, McFaul SJ, Ramasubramanian AK, Parida BK, Mora AG, Fedyk CG, et al. Primary hemostatic capacity of whole blood: a comprehensive analysis of pathogen reduction and refrigeration effects over time. Transfusion. 2013;53(Suppl 1):137S–49S.
39. Nair PM, Pandya SG, Dallo SF, Reddoch KM, Montgomery RK, Pidcoke HF, et al. Platelets stored at 4°C contribute to superior clot properties compared to current standard-of-care through fibrin-crosslinking. Br J Haematol. 2017;178(1):119–29.
40. Stolla M, Fitzpatrick L, Gettinger I, Bailey SL, Pellham E, Christoffel T, et al. In vivo viability of extended 4°C-stored autologous apheresis platelets. Transfusion. 2018;58(10):2407–13.
41. Reddoch-Cardenas KM, Bynum JA, Meledeo MA, Nair PM, Wu X, Darlington DN, et al. Cold-stored platelets: a product with function optimized for hemorrhage control. Transfus Apher Sci. 2019;58(1):16–22.
42. Holcomb JB, del Junco DJ, Fox EE, Wade CE, Cohen MJ, Schreiber MA, et al. The prospective, observational, multicenter, major trauma transfusion (PROMMTT) study: comparative effectiveness of a time-varying treatment with competing risks. JAMA Surg. 2013;148(2):127–36.
43. Glassberg E, Nadler R, Gendler S, Abramovich A, Spinella PC, Gerhardt RT, et al. Freeze-dried plasma at the point of injury: from concept to doctrine. Shock. 2013;40(6):444–50.
44. Moore HB, Moore EE, Morton AP, Gonzalez E, Fragoso M, Chapman MP, et al. Shock-induced systemic hyperfibrinolysis is attenuated by plasma-first resuscitation. J Trauma Acute Care Surg. 2015;79(6):897–903. discussion 903
45. Traverso LW, Lee WP, Langford MJ. Fluid resuscitation after an otherwise fatal hemorrhage: I. Crystalloid solutions J Trauma. 1986;26(2):168–75.
46. Pati S, Matijevic N, Doursout M-F, Ko T, Cao Y, Deng X, et al. Protective effects of fresh frozen plasma on vascular endothelial permeability, coagulation, and resuscitation after hemorrhagic shock are time dependent and diminish between days 0 and 5 after thaw. J Trauma. 2010;69(Suppl 1):S55–63.
47. Alam HB, Stanton K, Koustova E, Burris D, Rich N, Rhee P. Effect of different resuscitation strategies on neutrophil activation in a swine model of hemorrhagic shock. Resuscitation. 2004;60(1):91–9.
48. Deb S, Sun L, Martin B, Talens E, Burris D, Kaufmann C, et al. Lactated ringer's solution and hetastarch but not plasma resuscitation after rat hemorrhagic shock is associated with immediate lung apoptosis by the up-regulation of the Bax protein. J Trauma. 2000;49(1):47–53. discussion 53–5
49. Cardenas JC, Cap AP, Swartz MD, Huby MDP, Baer LA, Matijevic N, et al. Plasma resuscitation promotes coagulation homeostasis following shock-induced hypercoagulability. Shock. 2016;45(2):166–73.
50. Sperry JL, Guyette FX, Brown JB, Yazer MH, Triulzi DJ, Early-Young BJ, et al. Prehospital plasma during air medical transport in trauma patients at risk for hemorrhagic shock. N Engl J Med. 2018;379(4):315–26.
51. Moore HB, Moore EE, Chapman MP, McVaney K, Bryskiewicz G, Blechar R, et al. Plasma-first resuscitation to treat haemorrhagic shock during emergency ground transportation in an urban area: a randomised trial. Lancet. 2018;392(10144):283–91.
52. Matijevic N, Kostousov V, Wang Y-WW, Wade CE, Wang W, Letourneau P, et al. Multiple levels of degradation diminish hemostatic potential of thawed plasma. J Trauma. 2011;70(1):71–9; discussion 79–80.
53. Kakaiya RM, Morse EE, Panek S. Labile coagulation factors in thawed fresh frozen plasma prepared by two methods. Vox Sang. 1984;46(1):44–6.
54. Pandey S, Vyas GN. Adverse effects of plasma transfusion. Transfusion. 2012;52(Suppl 1):65S–79S.

55. Matijevic N, Wang Y-W, Cotton BA, Hartwell E, Barbeau JM, Wade CE, et al. Better hemostatic profiles of never-frozen liquid plasma compared with thawed fresh frozen plasma. J Trauma Acute Care Surg. 2013;74(1):84–90; discussion 90–91.
56. Como JJ, Dutton RP, Scalea TM, Edelman BB, Hess JR. Blood transfusion rates in the care of acute trauma. Transfusion. 2004;44(6):809–13.
57. Savage SA, Sumislawski JJ, Zarzaur BL, Dutton WP, Croce MA, Fabian TC. The new metric to define large-volume hemorrhage: results of a prospective study of the critical administration threshold. J Trauma Acute Care Surg. 2015;78(2):224–9. discussion 229–30
58. Anderson TN, Hinson HE, Dewey EN, Rick EA, Schreiber MA, Rowell SE. Early tranexamic acid administration after traumatic brain injury is associated with reduced syndecan-1 and angiopoietin-2 in patients with traumatic intracranial hemorrhage. J Head Trauma Rehabil. 2020;35(5):317–23.
59. Henry DA, Carless PA, Moxey AJ, O'Connell D, Stokes BJ, McClelland B, et al. Anti-fibrinolytic use for minimising perioperative allogeneic blood transfusion. Cochrane Database Syst Rev. 2007;4:CD001886.
60. Roberts I, Shakur H, Coats T, Hunt B, Balogun E, Barnetson L, et al. The CRASH-2 trial: a randomised controlled trial and economic evaluation of the effects of tranexamic acid on death, vascular occlusive events and transfusion requirement in bleeding trauma patients. Health Technol Assess. 2013;17(10):1–79.
61. Jehan F, Aziz H, O'Keeffe T, Khan M, Zakaria ER, Hamidi M, et al. The role of four-factor prothrombin complex concentrate in coagulopathy of trauma: a propensity matched analysis. J Trauma Acute Care Surg. 2018;85(1):18–24.
62. Kuckelman J, Barron M, Moe D, Lallemand M, McClellan J, Marko S, et al. Plasma coadministration improves resuscitation with tranexamic acid or prothrombin complex in a porcine hemorrhagic shock model. J Trauma Acute Care Surg. 2018 Jul;85(1):91–100.
63. Pati S, Potter DR, Baimukanova G, Farrel DH, Holcomb JB, Schreiber MA. Modulating the endotheliopathy of trauma: factor concentrate versus fresh frozen plasma. J Trauma Acute Care Surg. 2016;80(4):576–84; discussion 584–5.
64. Potter DR, Trivedi A, Lin M, Miyazawa BY, Vivona LR, McCully B, et al. The effects of human prothrombin complex concentrate on hemorrhagic shock-induced lung injury in rats: implications for testing human blood products in rodents. J Trauma Acute Care Surg. 2020;89(6):1068–75.
65. Franchini M, Lippi G. Fibrinogen replacement therapy: a critical review of the literature. Blood Transfus. 2012;10(1):23–7.
66. McRoyan DK, McRoyan CJ, Sauter KL, Liu PI, Daniel SJ. Antithrombin III, plasminogen, plasmin, and alpha-2-antiplasmin in donor blood and plasma components. Ann Clin Lab Sci. 1985;15(2):165–70.
67. Ketchum L, Hess JR, Hiippala S. Indications for early fresh frozen plasma, cryoprecipitate, and platelet transfusion in trauma. J Trauma. 2006;60(6 Suppl):S51–8.
68. Stensballe J, Ostrowski SR, Johansson PI. Haemostatic resuscitation in trauma: the next generation. Curr Opin Crit Care. 2016;22(6):591–7.
69. Barry M, Trivedi A, Miyazawa BY, Vivona LR, Khakoo M, Zhang H, et al. Cryoprecipitate attenuates the endotheliopathy of trauma in mice subjected to hemorrhagic shock and trauma. J Trauma Acute Care Surg. 2021;90:1022.
70. Ditillo M, Hanna K, Castanon L, Zeeshan M, Kulvatunyou N, Tang A, et al. The role of cryoprecipitate in massively transfused patients: results from the trauma quality improvement program database may change your mind. J Trauma Acute Care Surg. 2020;89(2):336–43.
71. Curry N, Rourke C, Davenport R, Beer S, Pankhurst L, Deary A, et al. Early cryoprecipitate for major haemorrhage in trauma: a randomised controlled feasibility trial. Br J Anaesth. 2015;115(1):76–83.
72. Schlimp CJ, Voelckel W, Inaba K, Maegele M, Ponschab M, Schöchl H. Estimation of plasma fibrinogen levels based on hemoglobin, base excess and Injury Severity Score upon emergency room admission. Crit Care. 2013;17(4):R137.
73. Hayakawa M, Maekawa K, Kushimoto S, Kato H, Sasaki J, Ogura H, et al. Hyperfibrinolysis in severe isolated traumatic brain injury may occur without tissue hypoperfusion: a retrospective observational multicentre study. Crit Care. 2017;23:21.
74. Nakamura Y, Ishikura H, Kushimoto S, Kiyomi F, Kato H, Sasaki J, et al. Fibrinogen level on admission is a predictor for massive transfusion in patients with severe blunt trauma: analyses of a retrospective multicentre observational study. Injury. 2017;48(3):674–9.
75. Rourke C, Curry N, Khan S, Taylor R, Raza I, Davenport R, et al. Fibrinogen levels during trauma hemorrhage, response to replacement therapy, and association with patient outcomes. J Thromb Haemost. 2012;10(7):1342–51.
76. Spahn DR, Bouillon B, Cerny V, Duranteau J, Filipescu D, Hunt BJ, et al. The European guideline on management of major bleeding and coagulopathy following trauma: fifth edition. Crit Care. 2019;23(1):98.
77. Schlimp CJ, Schöchl H. The role of fibrinogen in trauma-induced coagulopathy. Hamostaseologie. 2014;34(1):29–39.
78. Schöchl H, Nienaber U, Hofer G, Voelckel W, Jambor C, Scharbert G, et al. Goal-directed coagulation management of major trauma patients using thromboelastometry (ROTEM)-guided administration of fibrinogen concentrate and prothrombin complex concentrate. Crit Care. 2010;14(2):R55.
79. Nascimento B, Callum J, Tien H, Peng H, Rizoli S, Karanicolas P, et al. Fibrinogen in the initial resuscitation of severe trauma (FiiRST): a randomized feasibility trial. Br J Anaesth. 2016;117(6):775–82.
80. Hamada SR, Pirracchio R, Beauchesne J, Benlaldj MN, Meaudre E, Leone M, et al. Effect of fibrinogen concentrate administration on early mortality in traumatic hemorrhagic shock: a propensity score analysis. J Trauma Acute Care Surg. 2020;88(5):661–70.
81. Solomon C, Gröner A, Ye J, Pendrak I. Safety of fibrinogen concentrate: analysis of more than 27 years of pharmacovigilance data. Thromb Haemost. 2015;113(4):759–71.
82. Wikkelsø A, Wetterslev J, Møller AM, Afshari A. Thromboelastography (TEG) or thromboelastometry (ROTEM) to monitor haemostatic treatment versus usual care in adults or children with bleeding. Cochrane Database Syst Rev. 2016;8:CD007871.
83. Dhara S, Moore EE, Yaffe MB, Moore HB, Barrett CD. Modern management of bleeding, clotting, and coagulopathy in trauma patients: what is the role of viscoelastic assays? Curr Trauma Rep. 2020;6(1):69–81.
84. Moore HB, Gonzalez E, Moore EE. TEG/ROTEM-driven resuscitation in trauma. In: Martin Matthew J, Beekley Alec C, Eckert MJ, editors. Front line surgery: a practical approach. Cham: Springer; 2017. p. 595–610. https://doi.org/10.1007/978-3-319-56780-8_34.
85. Schöchl H, Cotton B, Inaba K, Nienaber U, Fischer H, Voelckel W, et al. FIBTEM provides early prediction of massive transfusion in trauma. Crit Care. 2011;15(6):R265.

41

Hemoglobin-Based Oxygen Carrier (HBOC) Development in Trauma: Previous Regulatory Challenges, Lessons Learned, and a Path Forward

Peter E. Keipert

Introduction

Hemoglobin-based oxygen carriers (HBOCs) have historically been referred to as "blood substitutes," which is a misnomer considering their transient circulatory half-life (i.e., typically ~24 h) versus transfused red blood cells (RBCs) that remain in circulation for weeks. As a result, there have been significant regulatory challenges to develop an HBOC as an alternative to RBC transfusion in clinical settings where blood transfusion is typically part of the standard of care. The risk of viral disease transmission from donor RBCs is always a concern to patients, even though the blood banking industry has implemented numerous strategies and new tests to reduce these risks to exceedingly low levels [1]. To market an HBOC for reduction or avoidance of RBC transfusion, regulators demanded compelling clinical data to demonstrate equivalent safety versus allogeneic donor RBCs. This would have required unrealistically large numbers of patients, and an unreasonable cost and time commitment. Commercial development therefore re-focused on alternative clinical scenarios where the physicochemical properties of the HBOC are used to enhance oxygen delivery to hypoxic tissues in ischemic organs. By acting as an "oxygen therapeutic" agent, the HBOC can be used as a "bridge" to transfusion, or as an alternative to RBCs in situations where transfusion is not an established therapy or may be contraindicated [2]. This provides a greater opportunity to prevent or treat ischemia-related morbidity and to potentially reduce mortality, and thereby demonstrate sufficiently compelling efficacy to satisfy the regulatory requirement for clinical benefit to gain marketing approval of an HBOC.

Medical Need in Trauma and Rationale for HBOCs

Mortality following blunt or penetrating injuries in trauma patients is typically caused by acute hemorrhagic shock resulting from severe and uncontrolled blood loss, and hemorrhage accounts for 30–40% of all deaths after trauma [3]. Shock is characterized by inadequate perfusion (ischemia) that leads to a shortage of cellular substrates, and insufficient oxygenation of vital organs (tissue hypoxia). Most tissues initially maintain adequate energy generation to support cellular metabolism by anaerobic glycolysis, resulting in lactic acid production in proportion to the overall oxygen debt. It is hypothesized that following severe shock, damage to the microcirculation delays restoration of normal perfusion, which is a significant contributor to worse outcome due to delayed resolution of the lactic acidosis.

Studies have shown that the severity of lactic acidosis in trauma is closely associated with worse outcomes [4, 5]. Moreover, the time needed to resolve lactic acidosis may be more important than the peak value reached, and prolonged elevation of lactate levels has been correlated with increased risk of organ failure and higher mortality [6]. Currently there is no approved medication or therapy to specifically address the altered state of the microcirculation during hemorrhagic shock. In order for an HBOC to perform as an "ischemic rescue" therapeutic agent, it must augment oxygen transport in the microcirculation, improve homogeneity of oxygen flux in capillaries, and facilitate diffusion of oxygen from RBCs to the endothelial cells lining vessels in hypoxic tissues.

The average mortality rate in modern trauma centers has reached low levels (~ 12–15%), which makes a pure mortality-based primary outcome for an HBOC trial a difficult hurdle to achieve. For those trauma patients in severe hemorrhagic shock who also exhibit higher levels of blood lactate (>5 mmol/L) due to critical tissue ischemia, a recent analysis by Lefering et al. (2013) showed that mortality rates are about two-fold higher (~ 25–30%) [7]. This higher-risk

P. E. Keipert (✉)
KEIPERT Corp. Life Sciences Consulting, San Diego, CA, USA

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_41

patient population therefore represents an opportunity for HBOC treatment, given as an adjunct to standard-of-care resuscitation, to improve survival as part of a composite clinical efficacy endpoint to demonstrate patient benefit in late-stage clinical trials.

Clinical Experience: Lessons Learned with HBOCs in Trauma

Several different HBOC formulations that demonstrated efficacy in preclinical animal models of hemorrhagic shock resuscitation, have also been tested in clinical trials in trauma: Baxter's α-α diaspirin crosslinked human Hb (*HemAssist*®), Biopure's glutaraldehyde-polymerized bovine Hb (*Hemopure*®), Northfield's glutaraldehyde-polymerized human Hb (*PolyHeme*®), and Sangart's maleimide- pegylated human Hb (MP4OX). The important lesson that has been learned in the past 10–15 years from these studies is that the physicochemical properties of each HBOC (e.g., lower P50, higher COP, degree of pegylation, type of chemical modification, molecular size, lower Hb concentration, physiological viscosity) play a role in determining both its safety profile, and whether a particular HBOC will enhance microvascular perfusion in critical organs and restore oxygenation of ischemic tissues sufficiently to reverse any accumulated oxygen debt. Despite success in preclinical animal models, several early-generation HBOC formulations (Baxter, Biopure, and Northfield) with higher P50 and lower COP were unable to translate their preclinical efficacy into patient benefit in late-stage human trauma trials [8].

One of the frequently observed adverse events (AEs) associated with earlier generation HBOCs was hypertension, caused by vasoconstriction secondary to scavenging of nitric oxide. This vasoconstriction effect had the unfortunate consequence of compromising the intended benefit of administering an HBOC to treat tissue ischemia in trauma. Perhaps more importantly, trial design issues played a role in limiting the ability of earlier HBOC trauma studies to demonstrate compelling clinical efficacy. By defining inclusion criteria that were overly broad, these studies allowed for inclusion of patients at both ends of the injury spectrum, i.e., those severely injured and likely to die no matter what intervention was given, and those with non life-threatening injuries who survive regardless of treatment. Using the degree of hypotension based on a patient's systolic blood pressure (SBP) < 90 mmHg as a criterion for inclusion may have limited proper evaluation of the severity of hemorrhagic shock, resulting in poor patient selection, potential under-resuscitation, and possibly inappropriate dosing. The Northfield study also withheld potentially life-saving RBC transfusions for 12 h post-injury. These protocol design issues represent shortcomings of the Baxter and Northfield trauma studies that may be partly to blame for their inability to demonstrate sufficient efficacy. When combined with the observations that the incidence of some serious adverse event (SAE) rates (including MI, hypertension, coagulopathy) and mortality were higher in HBOC-treated patients, it became impossible to achieve a favorable demonstration of clinical benefit and produced negative opinions and rejections from regulatory authorities. After their Phase 3 programs failed, both Baxter and Northfield decided to terminate their HBOC development due to insufficient funding to pursue new studies and based on other commercial and business considerations.

Sangart's recent Phase 2 trauma program with MP4OX added an important design feature missing from previous protocols, i.e., an elevated blood lactate ≥5 mmol/L as a physiological biomarker for inclusion at randomization to prospectively select patients who had suffered sufficient blood loss to reach a critical level of tissue ischemia [9]. Sangart completed a 51-patient pilot Phase 2a feasibility study in 2010 to compare two doses, and a 329-patient multicenter, randomized, single-dose Phase 2b study in 2012. Both of these studies were double blinded, and demonstrated the safety and potential efficacy of a low dose of MP4OX given as an adjunct to standard fluid resuscitation and blood product therapy (i.e., RBCs, FFP, platelets, as needed) in severely injured trauma patients. The Phase 2a study demonstrated more rapid reversal of lactic acidosis and a correlation with better outcomes when lactate levels normalized (≤2.2 mmol/L) within 8 h or when lactate decreased by ≥20% in 2 h [10]. In the Phase 2b study, a numerically higher percentage of patients treated with MP4OX were alive and discharged from hospital at Day 28 (primary efficacy endpoint) versus controls (57% vs. 50%; p = 0.18). Overall mortality in the MP4OX-treated patients was slightly lower compared to controls (11.6% vs. 13.9%; p = 0.73), which represents the first and only trauma study to demonstrate fewer deaths in HBOC-treated patients. There were no differences in the frequency of SAEs or AEs between the two groups. Multiple secondary endpoints also showed promising trends in the MP4OX group (i.e., fewer days on ventilator, in the ICU and in the hospital, as well as faster times to complete resolution of organ failure) [11].

In hindsight, the Phase 2b study was underpowered to confirm the efficacy of the 250-mL MP4OX treatment. One shortcoming of the Sangart Phase 2 program was the premature selection of a single low dose for the Phase 2b study, based on insufficient dose escalation to evaluate higher doses in the Phase 2a feasibility study, which resulted in the need for an additional follow-up study to complete a proper dose comparison. As a result, a 570-patient double-blind, controlled, Phase 2c dose-comparison protocol was designed to determine whether treatment with a 500-mL or 750-mL dose of MP4OX versus standard-of-care might improve patient

outcomes and demonstrate compelling efficacy in severely injured trauma patients with evidence of lactic acidosis due to hemorrhagic shock.

Regulatory Challenges, Safety, and a Pathway Forward

Previous regulatory guidance from the Food and Drug Administration (FDA) had suggested sponsors demonstrate safety of HBOCs in clinically stable elective surgery patients before moving to high-risk trauma patients [12]. Ironically, it is precisely in trauma patients where an HBOC with appropriate properties may have the greatest opportunity to show clinical benefit, as these patients present with a significant burden of morbidity and risk of death from severe hemorrhagic shock and the adverse ischemic consequences of tissue hypoperfusion. In April 2008, a conference sponsored by the FDA, the Dept. of Defense, and the National Institutes of Health (NIH) was convened to review the current status and safety of various HBOCs in development. The FDA's position and premise for holding this conference at that time was that similar SAE profiles amongst several HBOC products were raising questions regarding the possibility of common underlying mechanism(s) of toxicity despite differences between these HBOC formulations. A variety of opinions were expressed regarding these safety concerns; however, most experts suggested that the biggest challenge for the field was to identify appropriate clinical situations where there would be a more favorable balance of benefit to risk for HBOCs, and finding appropriate methods to evaluate the efficacy and safety of HBOCs in these settings [13].

Coincident with the FDA workshop, was the release of a publication by Natanson *et al*. reporting a meta-analysis to evaluate the relative risk of MI and death in patients enrolled in various HBOC trials [14]. Despite significant methodological issues and questionable statistical validity of a statistical analysis that aggregated disparate trials from a variety of patient populations (i.e., elective surgery vs. emergency or trauma), with different controls (patients receiving crystalloids vs. colloids vs. blood products), and HBOC preparations with diverse physicochemical properties, Natanson concluded that HBOCs were associated with an increased relative risk of death and MI. Due to concerns raised by this meta-analysis, the FDA imposed an immediate clinical hold on all HBOC trials that were either ongoing or planned in the USA at that time. Not surprisingly, several scientific experts and commercial HBOC developers challenged the validity of the methodology and conclusion(s) of this meta-analysis and the FDA's reaction to it [15].

Fortunately, regulatory authorities within the European Union (EU) and in multiple countries worldwide disagreed with the FDA-imposed moratorium, and decided to allow clinical studies to continue after performing their own internal safety review of all relevant clinical and preclinical data provided to them. At that time, Sangart decided to re-focus their clinical development from elective orthopedic surgery to a high-risk trauma indication where MP4OX would have a greater opportunity to demonstrate patient benefit, using Sangart's clinical data from two previous Phase 3 trials in hip arthroplasty [16, 17] as a supporting safety database. Regulators in South Africa, France, Germany, and the UK approved the Phase 2a pilot trauma study in 2009. Subsequently, from 2011 to 2012, Sangart also completed a larger Phase 2b trauma trial after successfully obtaining regulatory approvals in 14 countries worldwide (not including the USA).

While the FDA was intrigued by the success of the Phase 2a trial and the safety profile for MP4OX in that study, they were still unwilling to allow US sites to participate in the Phase 2b trauma trial. However, the FDA was eager for Sangart to show them the results from the Phase 2b study. To help the FDA understand that their pegylated and high affinity MP4OX formulation was different from most early-generation HBOCs, Sangart agreed with the FDA's request for a new submission to summarize all relevant biophysical characterization data, preclinical hemodynamic pharmacology and oxygenation findings, and any new clinical results available from the trauma program. A follow-up pre-IND submission for a new Phase 2c trauma study to compare two higher doses of MP4OX was submitted to the FDA in 2013, and the FDA agreed that Sangart could include US sites in this international trial.

Unfortunately, this positive opinion from the FDA came too late for Sangart to initiate patient enrollment in the Phase 2c study, because a failure to secure new funding forced Sangart to terminate development and cease operations in December 2013. Nevertheless, efforts are still underway to secure new funding to re-establish clinical development of MP4OX and other HBOC formulations for potential future applications in trauma and other ischemia-related clinical settings.

Future Directions and Indications for HBOC Development

There have been many potential clinical indications proposed for using HBOCs to prevent or treat acute ischemic conditions [18]. These cover a range of potential applications: (i) protection/maintenance of the functional integrity of vital organs at risk from various medical conditions and/or surgical procedures, e.g., brain (stroke, TBI), spinal chord (vascular surgery), heart (MI, cardiac arrest, angioplasty, CPR, bypass surgery), kidney (transplant surgery), and gut (surgery, shock); (ii) oncology applications to enhance oxy-

genation of solid tumors (during radiation and/or chemotherapy); (iii) organ transplantation (*ex vivo* perfusion to prolong storage hold-time for heart, lung, kidney, or liver), (iv) drug delivery (targeting oncology drugs conjugated to Hb to the liver); (v) ischemic limbs (peripheral vascular disease, diabetes); (vi) wound healing; (vii) Sickle Cell Disease (acute vaso-occlusive crisis); sepsis (refractory hypotensive shock); (ix) acute hemolytic anemia (oxygenation bridge); (x) veterinary use (due to limited availability of species-specific animal blood); and (xi) compassionate use (as a temporary blood substitute) for life-threatening anemia when RBCs are not available.

A number of ischemia-related indications were being pursued by Prolong Pharmaceuticals (South Plains, NJ) in early-stage clinical trials using their pegylated bovine carboxyHb (*Sanguinate*™). By correcting oxygenation levels and down-regulating inflammation, *Sanguinate* may have the potential to effectively treat many of the debilitating comorbidities of Sickle Cell disease (SCD) and other disorders caused by anemia and/or hypoxia/ischemia [19]. These include preventing delayed graft function following kidney transplantation, treating painful vaso-occlusive crises in adult patients with SCD and beta-Thalassemia, and reducing or preventing delayed cerebral ischemia following subarachnoid hemorrhage.

Recent research in the field has focused on the oxidative properties of Hb and how they can be modified to reduce the potential intrinsic toxicity of exogenously added iron and heme when HBOCs are infused. One such approach, developed by SynZyme Technologies (Sioux Falls, SD) [20] involves polynitroxylation of a pegylated Hb to create PNPH (aka *SanFlow*, or nanoRBC) as a nanomedicine for use in critical care and resuscitation following hemorrhagic shock [21]. Polynitroxylation adds superoxide dismutation activities to the Hb, which when infused create a superoxide-free vascular space. This helps to conserve endogenous nitric oxide levels within the vasculature, thereby preventing vasoconstriction and maintaining microcirculatory blood flow. PNPH has been evaluated in preclinical animal studies in stroke [22] and traumatic brain injury (TBI) [23], and has demonstrated both safety and neuroprotective properties (i.e., prevent neuronal death, restore MAP, reduce brain edema and increase cerebral perfusion pressure) following resuscitation in a murine model of combined TBI plus hemorrhagic shock [24].

Traumatic hemorrhage from penetrating and/or blunt injury offers a huge potential market for an HBOC as an adjunct to early resuscitation in both military and civilian settings. Similarly, non-traumatic hemorrhagic shock represents a potential expansion of the trauma indication, by using HBOC treatment following cerebral bleeding, ruptured aortic aneurysms, iatrogenic hemorrhage during vascular surgery, or obstetric bleeding from a ruptured placenta. TBI represents a subset of trauma with perhaps the highest mortality and longest hospital and ICU stays. Any HBOC that can treat oxidative stress from superoxide, down-regulate inflammation, and deliver oxygen to ischemic tissue holds great promise as a potential therapeutic agent in trauma, TBI, SCD, and possibly in stroke.

Acknowledgments PEK was formerly employed by Sangart Inc. (San Diego, CA) as VP, Clinical Development, during the conduct of the MP4OX clinical trials in trauma.

References

1. Epstein JS, Jaffe HW, Alter HJ, et al. Blood system changes since recognition of transfusion-associated AIDS. Transfusion. 2013;53:2365–74.
2. Mitchell P, Weiskopf R, Zapol WM. NIH/FDA/DOD interagency working group on oxygen therapeutics. In: Kim HW, Greenburg AG, editors. Hemoglobin-based oxygen carriers as red cell substitutes and oxygen therapeutics. Berlin: Springer; 2013. p. 141–7.
3. Kauvar DS, Wade CE. The epidemiology and modern management of traumatic hemorrhage: US and international perspective. Crit Care. 2005;9:S1–9.
4. Abramson D, Scalea TM, Hitchcock R, et al. Lactate clearance and survival following injury. J Trauma. 1993;35:584–9.
5. Manikis P, Jankowski S, Zhang H, et al. Correlation of serial blood lactate levels to organ failure and mortality after trauma. Am J Emerg Med. 1995;13:619–22.
6. Regnier MA, Raux M, Le Manach Y, et al. Prognostic significance of blood lactate and lactate clearance in trauma patients. Anesthesiology. 2012;117:1276–88.
7. Lefering R, Zielske D, Bouillon B, et al. Lactic acidosis is associated with multiple organ failure and need for ventilator support in patients with severe hemorrhage from trauma. Eur J Trauma Emerg Surg. 2013;39(5):487–93. https://doi.org/10.1007/s00068-013-0285-3.
8. Jahr JS, Akha AS, Holtby RJ. Crosslinked, polymerized, and PEG-conjugated hemoglobin-based oxygen carriers: clinical safety and efficacy of recent and current products. Curr Drug Discov Technol. 2012;9:158–65.
9. Brohi K, Plani F, Moeng M, et al. Multicenter randomized controlled trial TRA-204 to evaluate the safety and efficacy of MP4OX in lactate clearance following trauma hemorrhage. Crit Care Med. 2010;38:749.
10. Brohi K, Levy H, Keipert PE, et al. Normalization of lactate within 8 hours or ≥20% clearance in initial 2 hours correlate with outcomes from traumatic hemorrhage. Crit Care Med. 2011;39:643.
11. Brohi K, Boffard K, Zielske D, et al. Effects of MP4OX, an oxygen therapeutic, on clinical outcomes in trauma patients with hemorrhagic shock: a phase IIb multi-center randomized placebo-controlled trial. Poster presentation: American Association for the Surgery of Trauma (AAST) meeting, San Francisco. 2013.
12. US Food and Drug Administration. Draft guidance for industry: criteria for safety and efficacy evaluation of oxygen therapeutics as red blood cell substitutes. Issued Oct 2004 www.rsihata.com/updateguidance/usfda2/bio1/ucm080290. 2004.
13. Silverman TA, Weiskopf RB. Hemoglobin-based oxygen carriers: current status and future directions. Anesthesiology. 2009;111:946–63.
14. Natanson C, Kerns SJ, Lurie P, et al. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death. A meta-analysis. JAMA. 2008;299:2304–12.

15. Letters to the Editor (2008) Hemoglobin-based blood substitutes and risk of myocardial infarction and death. JAMA 300:1295–1300.
16. Olofsson CI, Górecki AZ, Dirksen R, et al. Evaluation of MP4OX for prevention of perioperative hypotension in patients undergoing primary hip arthroplasty with spinal anesthesia: a randomized, double-blind, multicenter study. Anesthesiology. 2011;114:1048–63.
17. Van der Linden P, Gazdzik TS, Jahoda D, et al. A double-blind, randomized, multicenter study of MP4OX for treatment of perioperative hypotension in patients undergoing primary hip arthroplasty under spinal anesthesia. Anesth Analg. 2011;112:759–73.
18. Winslow RM. Clinical indications for blood substitutes and optimal properties. In: Winslow RM, editor. Blood substitutes. London: Elsevier; 2006. p. 115–25.
19. Misra H, Lickliter J, Kazo F, et al. Pegylated carboxyhemoglobin bovine (*Sanguinate*): results of a phase I clinical trial. Artif Organs. 2014;38:702–7.
20. Hsia JC, Ma L. Compositions and methods of use of neurovascular protective multi- functional polynitroxylated pegylated carboxy hemoglobins for transfusion and critical care medicine. 2012. US Patent 8273857B2 (https://patents.google.com/patent/US8273857B2/en).
21. Hsia CJC, Ma L. A hemoglobin-based multifunctional therapeutic: polynitroxylated pegylated hemoglobin. Artif Organs. 2012;36:215–20.
22. Zhang J, Cao S, Ma L, et al. Protection from transient focal cerebral ischemia by transfusion of polynitroxylated pegylated hemoglobin. Stroke. 2013;44:A154.
23. Shellington DK, Du L, Wu X, et al. Polynitroxylated pegylated hemoglobin: a novel neuroprotective hemoglobin for acute volume-limited fluid resuscitation after combined traumatic brain injury and hemorrhagic hypotension in mice. Crit Care Med. 2011;39:494–505.
24. Brockman EC, Bayir H, Blasiole B, et al. Polynitroxylated-pegylated hemoglobin attenuates fluid requirements and brain edema in combined traumatic brain injury plus hemorrhagic shock in mice. J Cereb Blood Flow Metab. 2012;33:1457–64.

Use of Oxygen Therapeutics in Patients for Whom Blood Is Not an Option

42

Aryeh Shander, Sherri Ozawa, and Mazyar Javidroozi

Background

Patients for whom transfusion of blood components is not an option represent a diverse group. Some patients refuse transfusions due to personal convictions, religious beliefs or concerns over risks and complications of blood [1, 2]. There are patients who cannot be transfused due to medical reasons such as presence of alloantibodies and increased risk of hemolytic reactions [3]. Additionally, unavailability of appropriate cross-matched blood units due to logistical constraints, shortages, disasters and disruptions in blood supply and distribution can effectively lead to scenario in which a patient in need of transfusion must be managed without allogeneic blood [2, 4].

The scientific and medical community owes a substantial part of its knowledge and expertise on treating severely anemic patients without transfusion to those who belong to the Jehovah's Witness faith. Based on their interpretation of various Biblical passages (e.g. Genesis 9:4 and Leviticus 17:10), blood is considered sacred and abstaining from blood consumption (including transfusion) is a key principle of Jehovah's Witness faith [5]. At times, this conviction has placed patients and their clinicians in a difficult situation in which a clinician had to refrain from administrating a seemingly lifesaving treatment to a gravely ill patient.

Nonetheless, refusing one treatment modality should not be equated with refusing medical care altogether, and many other treatment modalities are often acceptable to these patients [2]. In response to the important challenge of providing effective care without violating patients' values and maintaining their right of self-determination, various medical strategies have been developed that allow patient care without the use of allogeneic blood components. Known collectively as Bloodless Medicine and Surgery, these strategies paved the way for development of the concept of Patient Blood Management which aims to improve the outcome of all patients (regardless of their view on transfusions) through the timely application of various evidence-based medical and surgical strategies to maintain hemoglobin concentration, support hemostasis, minimize blood loss and maximize oxygen delivery to tissues [4, 6, 7].

While many of these strategies predominantly place emphasis on preventive measures to avoid severe anemia that is likely to result in the need for transfusion (e.g. timely diagnosis and management of anemia before it gets too severe and prevention of blood loss), there are also treatment options that can be used in severe anemia to maintain oxygen delivery, support circulation, improve perfusion and reduce tissue ischemia and injury [2, 8–10]. Oxygen therapeutics such as hemoglobin-based oxygen carriers (HBOCs) are among the treatments that have been used in this context in patients for whom blood transfusion is not an option.

Expanded Access and Right to Try

No HBOC products is approved for clinical use in the US and most other countries across the world (with the exception of South Africa and Russia) [11]. As such, access to these products is often limited and only possible through clinical trial protocols designed to study their safety and efficacy.

In the US, the Food and Drug Administration (FDA) has devised a process called Expanded Access (also known as "Compassionate Use") to streamline the access to investigational products in a timely manner outside of clinical trials for patients with an immediately life-threatening condition or serious disease, when no satisfactory alternative treatment option is available [12]. The investigational treatment can be

A. Shander (✉) · M. Javidroozi
Department of Anesthesiology, Critical Care and Hyperbaric Medicine, Englewood Hospital and Medical Center, Englewood, NJ, USA
e-mail: aryeh.shander@ehmchealth.org

S. Ozawa
Institute for Patient Blood Management and Bloodless Medicine and Surgery, Englewood Health, Englewood, NJ, USA
e-mail: sozawa@accumen.com

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_42

Table 42.1 Patient conditions that should be met in order to consider expanded access or right to try for an investigational treatment according to the FDA

Expanded Access	Right to Try
Patient has a serious disease or condition, or whose life is immediately threatened by their disease or condition.	Patient has been diagnosed with a life-threatening disease or condition.
There is no comparable or satisfactory alternative therapy to diagnose, monitor, or treat the disease or condition.	Patient has exhausted approved treatment options.
Patient enrollment in a clinical trial is not possible.	Patient is unable to participate in a clinical trial involving the eligible investigational drug.
Potential patient benefit justifies the potential risks of treatment.	
Providing the investigational medical product will not interfere with investigational trials that could support a medical product's development or marketing approval for the treatment indication.	The investigational drug under consideration has completed a phase 1 clinical trial, has not been approved or licensed by the FDA for any use, is under investigation in a clinical trial that is intended to form the primary basis of a claim of effectiveness in support of FDA approval and is the subject of an active investigational new drug application submitted to the FDA; or its active development or production is ongoing, and that has not been discontinued by the manufacturer or placed on clinical hold by the FDA
Patient or patient's legally authorized representative has provided a written informed consent	

https://www.fda.gov/news-events/public-health-focus/expanded-access and https://www.fda.gov/patients/learn-about-expanded-access-and-other-treatment-options/right-try [last accessed on 06/12/2021]

provided under the Expanded Access program in one of the three categories: For individual patients (including for Emergency Use); for intermediate-size patient groups; and for widespread treatment use. FDA has provided guidelines to determine when Expanded Access to an investigational treatment may be considered (Table 42.1) [13].

The Emergency Use option under the single-patient Expanded Access category is intended to facilitate the use of investigational treatment when time is of the essence. The process starts by the treating physician making an emergency request (e.g. through a phone call or another rapid communication method) to the FDA with the request to use the investigational therapy. The request is reviewed expeditiously by an FDA medical officer and upon approval and authorization (which can be verbal), the manufacturer is notified to arrange for delivery of the medication/device. If there is no sufficient time to obtain Institutional Review Board (IRB) approval prior to the first use of the investigational treatment in the institution, treatment may be provided without prior IRB review and only with approval under Emergency Use, provided that it is reported to the IRB within 5 days. Any unused medication/device should be destroyed or returned to the manufacturer and this process must be started "from scratch" for every new patient [14].

In contrast, the second category of Expanded Access (for intermediate-size patient groups) can provide a more practical and streamlined process for making the investigational treatment available to patients in need, provided that proper planning and preparations have been made in advance. Under this category, an institution which is likely to encounter several patients who can be candidates for a specific treatment under Expanded Access program submits an Investigational New Drug (IND) application and protocol to the FDA and its IRB. Once the protocol is approved and IND number is issued by the FDA, the manufacturer will be able to ship the investigational product to the institution to maintain an in-house stock to be used for future patients meeting the criteria of the Expanded Access protocol. This process can avoid the delays caused by the requirement to file multiple INDs and shipping investigational medication for individual patients [14].

Recently, another avenue to provide access to unapproved drugs (not devices) has become available through the federally-legislated "Right to Try" act [15]. The patient criteria to be considered for this program are similar to those of Expanded Access (presence of a life-threatening condition, exhaustion of approved treatment options, inability to participate in a clinical trial for the investigational treatment, and consenting to treatment). The investigational drug to be considered under this program should have completed a Phase 1 clinical trial and should be under active development or production and subject of an active investigational new drug (IND) application submitted to the FDA. Unlike the Expanded Access program, the Right to Try program is only available to single patients, but it provides a streamlined process to grant access to investigational drugs to patients without FDA oversight. While the current federal Right to Try laws do not require IRB review, some states' Right-to-Try access laws requires IRB oversight [15].

The policies and requirements of IRBs for Expanded Access and Right-to-Try may vary significantly at various institutions. Surveys have pointed out to variations in the requirements for full IRB reviews versus review by IRB chairs or individual members, timeline of the review process, policies related to financial aspects of the treatments and permitted uses of clinical data from each case. Furthermore, identifying, understanding and interpreting IRB policies and regulations can be complicated, and even more challenging in cases of Expanded Access when clinicians are pressed for time, trying to expedite the care of their patients [16]. For these reasons, it is important that clinicians who are likely to see patients who might be candidates for Expanded Access or Right-to-Try, should engage in discussions with their

IRBs ahead of time to identify the process and understand the requirements to avoid potential delays or other issues when the need arrives.

Reports of Use of HBOCs Under Expanded Access Programs

HBOC products have been used under Expanded Access program for years in management of severely anemic patients when transfusion is indicated but not possible [14]. The goal is often to sustain the oxygen delivery to the tissues above the critical level needed to avoid tissue ischemia and injury and to buy some time for other treatments of anemia (such as erythropoiesis stimulating agents) to exert their effects and raise hemoglobin concentration. In other words, these agents act as "oxygen bridges" or "bridging treatments" to sustain the patients until their endogenous hemoglobin concentration has recovered [17–20]. To date, there is no reported case of Right-to-Try use for these products. The Expanded Access program has provided us with invaluable insight into the clinical use of these medications and has possibly contributed to patients surviving dire and life-threatening conditions [18].

The HBOCs used under the Expanded Access program include Hemopure® (HBOC-201; hemoglobin glutamer – 250 [bovine]) and Sanguinate® (PEGylated bovine carboxyhemoglobin). Table 42.2 provides a summary of key characteristics of these products and they are discussed in detail in their respective chapters here [11, 18, 21, 22].

Mackenzie et al. reported 54 patients with life-threatening anemia (median hemoglobin 4 g/dL at time of HBOC request) for whom transfusion was not an option who were treated with Hemopure (60 to 300 g) across several institutions [23]. The main causes of anemia in this case series were surgical blood loss (45%), malignancy (18%) and hemolysis (13%). Twenty three patients (41.8%) survived. Increased duration and severity of anemia, delayed HBOC infusion, and presence of malignancy or renal disease were associated with increased risk of mortality. There were no serious adverse events linked to Hemopure. The authors concluded that based on their findings, earlier treatment with HBOC is likely to lead to better survival [23].

Donahue et al. reported a Jehovah's Witness patient who developed severe symptomatic anemia with hemoglobin reaching 3.1 g/dL following induction chemotherapy for acute lymphoblastic leukemia (ALL) [17]. The patient received 15 units of Hemopure over 12 days. During this period, total hemoglobin concentration was maintained between 3.6 and 5.3 g/dL and no evidence of organ ischemia or dysfunction was noted. The patient was able to complete the chemotherapy regimen and was discharged in stable condition [17].

Fitzgerald et al. reported a Jehovah's Witness patient with multiple traumatic injuries who developed severe anemia (hemoglobin 2.9 g/dL by the fifth day of admission) with evidence of cardiac hypoxia (increased troponin I, widespread ST depression and a documented episode of ventricular tachycardia) following significant blood loss and fluid resuscitation. The patient underwent slow infusion of 5 units of Hemopure over days 5 and 6, and patient's hemoglobin increased to 6.2 g/dL and troponin I levels and electrocardiography results normalized, eventually allowing the patient to undergo fracture fixation. The patient was discharged with recovered hemoglobin concentrations and in well condition [24]. In another trauma case report, a Jehovah's Witness patient experiencing hemorrhagic shock due to acute blood loss with a nadir hemoglobin concentration of 3.9 g/dL was successfully managed

Table 42.2 Key characteristics of Hemopure® (HBOC-201; hemoglobin glutamer – 250 [bovine]) and Sanguinate® (PEGylated bovine carboxyhemoglobin), the HBOCs used on expanded access basis [11, 18, 21, 22]

	Hemopure®	Sanguinate®	Human packed RBCs
Biochemical modifications	Purified, cell-free, glutaraldehyde crosslinked polymerized hemoglobin	Purified, cell-free, PEGylated (i.e. conjugated with polyethylene glycol) carboxyhemoglobin	None
Source of hemoglobin	Bovine	Bovine	Human
Molecular weight (kD)	87–500	120	64
HBOC concentration (g/dL)	12–14	3–4	13
Oncotic pressure	Normo-oncotic	Hyper-oncotic	Normo-oncotic
Unit size	250 mL	500 mL	Varies
P50 (mmHg)	40	7–15	26
Circulatory half-life (hours)	18–24	13–20 (lower in healthy volunteers, higher in sickle cell anemia patients)	Several hundred hours
Cross-matching required?	No	No	Yes
Oxygen affinity affected by 2,3-DPG?	No	No	Yes
Storage and shelf-life	Up to 3 years at room temperature	Up to 2 years at room temperature	Up to 4 days at 4 degrees centrigrade

and sustained with a multifaceted treatment strategy including early infusion with a HBOC until endogenous hemoglobin concentration raised sufficiently [25].

Jordan and Alexander reported a Jehovah's Witness patient who presented with autoimmune hemolytic anemia [26]. The patient received treatment to control the hemolytic anemia but the hemoglobin continued to drop from 8.4 g/dL at admission to lowest concentration of 2.8 g/dL on the fourth day. After receiving 2 units of Hemopure, the patient's hemoglobin concentration increased to 8.7 g/dL, her clinical condition improved and she was subsequently discharged [26].

Lundy et al. reported an elderly Jehovah's Witness patient who had suffered extensive full-thickness burn [27]. Following severe anemia with evidence of critically low oxygen delivery during the perioperative period of surgical excision, the patient received 6 units of Hemopure during the perioperative period. No adverse effect was noted, but the patient eventually died due to progressively worsening multiple organ failure [27].

Thenuwara et al. reported a case of Jehovah's Witness patient whose hemoglobin dropped from 12 g/dL to 3 g/dL within one day following cesarean section and postpartum hemorrhage, leading to acidosis [28]. The patient received supportive care (vasopressors, sedation and ventilation) and was treated with daily erythropoietin, iron and vitamin B12 but as the bleeding continued, she was treated with Sanguinate under Expanded Access program. Her hemoglobin increased to 7 g/dL by the day 8 after surgery and she became hemodynamically stable with no need to vasopressors and she was extubated and discharged from the ICU [28].

Sam et al. reported the first case of a Jehovah's Witness patient with thrombotic thrombocytopenic purpura (TTP) who could not undergo therapeutic plasma exchange and was treated with Sanguinate [29]. The patient tolerated the treatment well, and the hemoglobin concentration and platelet count improved leading to the patient's discharge [29].

Davis et al. described three critically ill patients for whom blood transfusion was not an option with multi-organ failure during sickle cell crisis [30]. The patients received between 6 and 27 units of Hemopure (setting a record for the largest volume of Hemopure infused to a patient) and all three cases were discharged from the hospital [30].

DeSimone et al. reported a 42-year-old Jehovah's Witness patient who presented with acute upper gastrointestinal (GI) bleeding leading to hemorrhagic shock [31]. The patient received 6 units of Sanguinate alongside with other supportive care and treatment for anemia. The patient's shock and encephalopathy improved, and the patient was successfully extubated [31].

As indicated earlier, patients for whom blood transfusion is not an option represent a diverse group and not all cases are related to religious beliefs. Unnikrishnan et al. reported a patient with sickle cell anemia who developed an alloantibody-mediated life-threatening delayed hemolytic reaction following blood transfusion for acute chest syndrome [32]. The patient received Hemopure alongside with other treatments for hemolytic anemia [32].

Brotman et al. treated a 77-year-old Jehovah's Witness patient who developed severe symptomatic anemia (hemoglobin 4.5 g/dL) following cystoprostatectomy and radical nephrectomy with Sanguinate [33]. The patient's anemia improved and he was discharged to a rehabilitation facility [33]. Sanguinate has also been used successfully in treatment of a Jehovah's Witness patient undergoing liver transplant [34].

Zumberg et al. reported 10 severely anemic cases for whom blood was not an option who received high dose of Hemopure (defined as 10 units or more during hospital stay) [14]. The etiology of anemia in these patients included malignancy, sickle cell crisis, autoimmune hemolysis, red cell aplasia, post-partum hemorrhage, postoperative hemorrhage and GI bleeding. Patients had a median pre-infusion nadir hemoglobin concentration of 3.3 g/dL and they received an average of 16.2 units of Hemopure. Following Hemopure treatment, their median hemoglobin was 7.3 g/dL. The authors reported side effects including methemoglobinemia, GI symptoms and hypertension but they did not report any serious adverse events and all patients survived [14].

In a case series that can highlight the limitations of use of HBOC product under current Expanded Access program, McConachie et al. reported 3 Jehovah's Witness patients with severe anemia (hemoglobin <5 g/dL) who were considered for treatment with Sanguinate under the Emergency Use program [35]. One patient died while waiting but the two others received the product. Among them, one died after receiving 5 units of HBOC and the other patient recovered and was discharged [35].

Mackenzie et al. have provided an account of the lessons learned during treatment of over 1700 patients with Hemopure under clinical trials and CU programs [36]. They listed increased blood pressure, oliguria, GI symptoms, yellow discoloration of sclera and skin and transient increase in blood methemoglobin and liver/pancreatic enzymes as the main reported adverse events and indicated that most were transient, self-limiting or easily manageable. The authors noted that issues related to improper consideration of the volume expanding effects of Hemopure and its half-life in the body which necessitates repeated infusions were the most common clinical management errors. They also underscored the importance of early use of HBOC under CU programs as soon as hemoglobin drops below 5 g/dL to improve survival [36].

The impact of HBOC infusion goes far beyond the immediate effect on hemoglobin concentration. As a matter of fact and as can be seen in Table 42.2, Sanguinate units have a hemoglobin concentration of less than 4 g/dL. Therefore, infusion of Sanguinate in a patient with hemoglobin concentration of over 4 g/dL can initially lead to hemodilution and further drop in hemoglobin concentration [18, 37]. How severely anemic patients could benefit from this product can be explained by considering other characteristics of Sanguinate. Namely, the product has a low p50, which can assist in transferring oxygen from RBCs into ischemic tissues with very low partial oxygen pressure. The smaller molecular weight of the Sanguinate and its dual role as oxygen and carbon monoxide transfer agent might further assist oxygen delivery in hypoxic and ischemic tissues while reducing inflammation [18, 37]. The possibility of HBOCs further assisting hematopoiesis by providing bioavailable iron should also be considered.

Currently there are four active Expanded Access programs for HBOCs available and listed under ClinicalTrials.gov. They offer Hemopure to treat life-threatening anemia in patents for whom blood transfusion is not an option (Table 42.3).

Summary and Lessons Learned from the Use of HBOCs Under Expanded Access Programs

No medical treatment is without risks and practice of medicine revolves around balancing the purported benefits of the treatments versus their potential risks in an effort to improve the outcomes of patients without violating personal choices and values. HBOCs are no exception to this principle. Earlier trials of HBOCs were remarkable for observed complications including increased risk of myocardial infarction and death [38], leading to calls for moratorium on any further trials on these products [39]. What was left out in these arguments was the scenarios in which patients might be facing risks much graver that the risks of HBOCs with no other alternative treatments available.

Table 42.3 Active expanded access programs for HBOCs currently available under ClinicalTrials.gov.

Title	ClinicTrials.gov identifier	Start year	Product	Eligibility criteria	Exclusion criteria	Location
Expanded access study of HBOC-201 (Hemopure) for the treatment of life-threatening anemia	NCT01881503	2013	HBOC-201	Consenting patients ≥18 years old with Hb ≤8 g/dL with active bleeding, physiologic evidence of critical ischemia such as elevated troponins, altered mental status, acute renal failure, lactic acidosis or central nervous system supply dependency	Known hypersensitivity or allergy to beef products Pre-existing uncontrolled hypertension, heart failure, renal failure, circulatory hypervolemia or systemic mastocytosis[a] Eligibility for blood transfusions Age > 80 years old[a] ***Pregnancy or lactating***	Englewood Hospital and Medical Center, Englewood, NJ
Expanded access protocol using HBOC-201 to treat patients with life threatening anemia, for whom blood is not an option	NCT02684474	2016	HBOC-201	Consenting critically ill patients ≥18 years old with Hb ≤6 g/dL (or 7–8 g/dL with significant active bleeding), and physiologic evidence of critical ischemia, for example: elevated troponins, altered mental status, acute renal failure, lactic acidosis or evidence of central nervous system acute deficits, when blood is not an option due to refusal of transfusion or lack of compatible red blood cells		The Johns Hopkins Hospital, MD
HBOC-201 expanded access protocol for life-threatening anemia for whom allogeneic blood transfusion is not an option	NCT02934282	2016	HBOC-201	Consenting critically ill patients ≥18 years old with Hb <5 g/dL (or 6–7 g/dL with significant active bleeding), and physiologic evidence of critical ischemia, for example: elevated troponins, altered mental status, acute renal failure, lactic acidosis or evidence of central nervous system acute deficits, when blood is not an option due to refusal of transfusion or lack of compatible red blood cells		Jackson Memorial Hospital, Miami, FL
Expanded access IND administration of HBOC-201 in patients with severe acute anemia	NCT03633604	2018	HBOC-201	Consenting critically ill patients ≥18 years old with Hb ≤6 g/dL (or 7–8 g/dL with significant active bleeding), and physiologic evidence of critical ischemia, for example: elevated troponins, altered mental status, acute renal failure, lactic acidosis or evidence of central nervous system acute deficits, when blood is not an option due to refusal of transfusion or lack of compatible red blood cells		University of Pittsburgh Medical Center, Pittsburgh, PA

[a]On a case by case and quality of life determination basis

Current HBOCs might not be a match for allogeneic blood transfusions in terms of their safety and efficacy profile. However, HBOCs can offer an "oxygen bridge" for severely anemic critically ill patients who are candidate for blood transfusions but cannot be transfused for any reasons (logistical, medical or patient's personal preferences and convictions) and sustain them until their hematopoietic system can produce enough RBCs [20, 40].

As can be seen in the reports summarized here, in the absence of FDA-approved HBOCs or ongoing clinical trials, Expanded Access programs have provided a potentially life-saving option for critically-ill severely anemic patients who cannot be transfused. While these case series offer a glimpse of hope for patients and their care givers, they should be interpreted in light of their limitations.

Case series of therapeutics might be more vulnerable to various types of bias (e.g. selection, reporting and publication bias) and as such, they are considered to offer a lower class of evidence [41]. Without concurrent control groups, it can be difficult to interpret the findings and determine the net benefit (or harm) of HBOCs. Nonetheless, a few reports of outcomes of severely anemic patients who did not receive any transfusions or blood substitutes are available and can provide us with a picture of potential consequences of critical anemia faced by these patients [8, 42]: It is estimated that the mortality rate increases by up to 2–2.5 fold for every 1 g/dL drop in the hemoglobin concentration from a baseline range of 7–8 g/dL, reaching almost 100% at hemoglobin concentration drops below 2 g/dL [43]. Figure 42.1 depicts the probability of survival with various nadir postoperative hemoglobin concentrations in patients who were not transfused nor received HBOCs, based on our data [8] and the results reported by Carson et al. [42].

The lowest hemoglobin concentrations reported in the case series discussed here are around 3 g/dL which corresponds to a survival probability of around 50% according to the pooled data from Carson et al. and Shander et al. [8, 42] (Fig. 42.1). One might consider the reported survival of most patients in these HBOC case series against the expected survival rate of around 50% in this hemoglobin range as a partial victory, notwithstanding the possibility of bias in case series. On the other hand, the larger case series by Mackenzie et al. [23] reported an overall survival rate of 41.8% with a median hemoglobin concentration of 4 g/dL at time of requesting the HBOC product (which corresponds to an expected probability of survival of 70% according to the Carson and Shander data (Fig. 42.1). They did not report the nadir hemoglobin concentrations in their case series and hence it is likely that their patients had a lower expected survival probability according to their nadir hemoglobin concentration. Additionally, they reported a significantly longer duration of time between first known hemoglobin concentration < 8 g/dL and the start of the HBOCs in patients who did not survive versus those who survived (median 8 vs. 4 days)

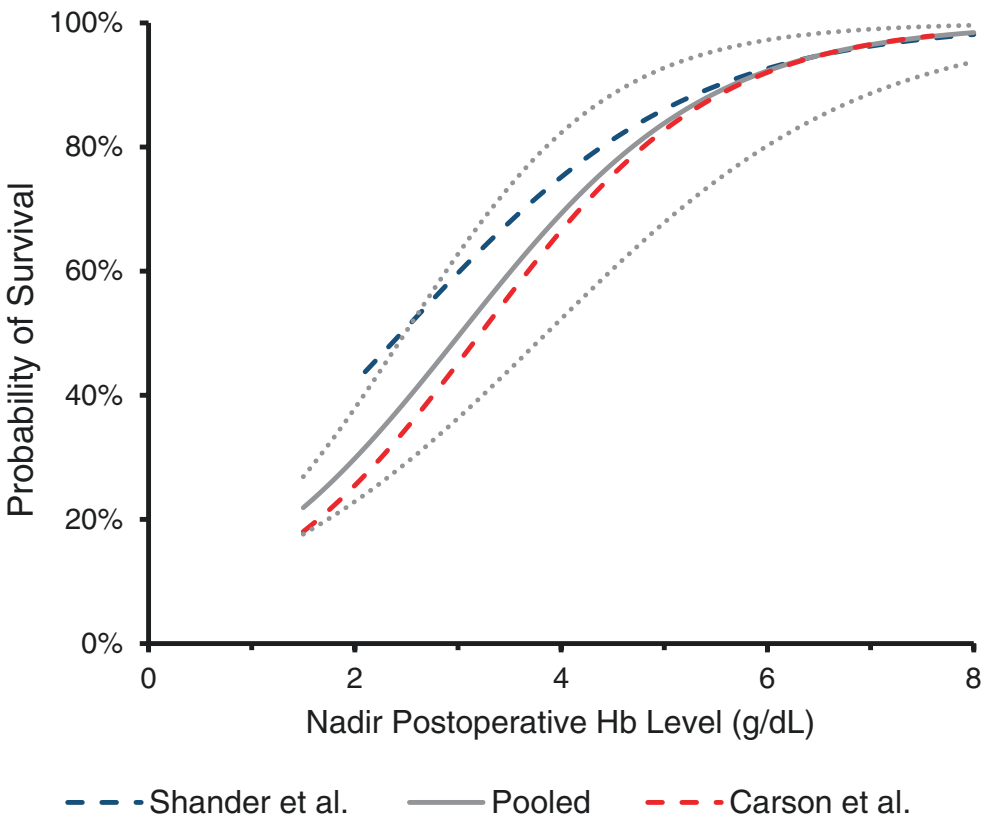


Fig. 42.1 Nadir postoperative hemoglobin concentrations and probability of survival in patients who were not treated with blood transfusion or HBOC products based on the reports by Carson et al. [42] (dashed red line) and Shander et al. [8] (dashed blue line) as well as the results of the two reports combined (gray solid line with 95% confidence intervals represented with dotted gray lines)

as well as variations in comorbidities (with none of the cancer patients surviving) [23]. These observations remind us of the difficulties of predicting the expected baseline survival rate in these patient populations without the use of HBOCs, which makes it difficult to determine the impact of HBOC on their outcomes.

Going through Expanded Access programs takes extra precious time during a critical period where every minute does count. As can be seen from the study by Mackenzie et al., likelihood of survival decreases as the duration of the time patients have suffered from severe anemia increases and it is not uncommon that days pass before the patient can receive the blood substitute. As previously indicated, the Expanded Access program for intermediate-sized patient group can facilitate the process by allowing the product stocked at the participating centers [23].

As discussed earlier, efforts to provide effective care for patients for whom transfusion was not an option led to the development of Bloodless Medicine and Surgery programs (and eventually, Patient Blood Management) [6]. For best outcomes, HBOCs should be used as part of comprehensive management protocols including several other strategies that increase oxygen delivery (e.g. supplemental oxygen and hyperbaric oxygen therapy), reduce oxygen demand (e.g. muscle blockers, maintenance of normovolemia, and avoidance of fever), promote hematopoiesis (e.g. using erythropoiesis stimulating agents and iron), manage underlying causes of anemia and reduce further blood loss (including iatrogenic blood losses) [2, 4, 6, 7, 19, 44–46]. A proactive and preventive approach to optimize hemoglobin concentration during the preoperative period to better prepare patients for whom transfusion is not an option for potential surgical blood loss is critical and protocols to achieve this goal are suggested [19]. The case reports discussed here all provide examples of several of these strategies that have been used successfully alongside with HBOCs to improve the outcomes of patients.

Key Points

- Patients for whom transfusion of blood components is not an option represent a diverse group including those who refuse transfusions due to personal convictions, religious beliefs or concerns over risks of blood, those who cannot be transfused due to medical reasons (e.g. alloantibodies) or logistical issues.
- Current HBOCs might not be a match for allogeneic blood transfusions in terms of their safety and efficacy profile, but they can offer an "oxygen bridge" for severely anemic critically ill patients who are candidate for blood transfusions but cannot be transfused for any reasons.
- In the US, HBOCs are available to these patients under the Expanded Access (or "Compassionate Use") program that has been devised by the FDA to streamline the access to investigational products in a timely manner outside of clinical trials for patients with an immediately life-threatening condition or serious disease, when no satisfactory alternative treatment options is available.
- Several case series describing the use of HBOCs under the Expanded Access program have been published, and they generally support the feasibility of the treatment, limited and well-tolerated side effects, and possible contribution to improve outcome of the patients.
- For best results, HBOCs should be used as part of comprehensive Patient Blood Management protocols including several other strategies aimed at increasing oxygen delivery, reducing oxygen demand, promoting hematopoiesis, managing underlying causes of anemia and reducing further blood loss.

References

1. Shander A, Javidroozi M. The approach to patients with bleeding disorders who do not accept blood-derived products. Semin Thromb Hemost. 2013;39(2):182–90.
2. Shander A, Goodnough LT. Management of anemia in patients who decline blood transfusion. Am J Hematol. 2018;93(9):1183–91.
3. Chonat S, Arthur CM, Zerra PE, Maier CL, Jajosky RP, Yee MEM, et al. Challenges in preventing and treating hemolytic complications associated with red blood cell transfusion. Transfus Clin Biol. 2019;26(2):130–4.
4. Shander A, Goobie SM, Warner MA, Aapro M, Bisbe E, Perez-Calatayud AA, et al. Essential role of patient blood management in a pandemic: a call for action. Anesth Analg. 2020;131(1):74–85.
5. Shander A, Goodnough LT. Objectives and limitations of bloodless medical care. Curr Opin Hematol. 2006;13(6):462–70.
6. Shander A, Javidroozi M, Perelman S, Puzio T, Lobel G. From bloodless surgery to patient blood management. Mt Sinai J Med. 2012;79(1):56–65.
7. Shander A, Bracey AW Jr, Goodnough LT, Gross I, Hassan NE, Ozawa S, et al. Patient blood management as standard of care. Anesth Analg. 2016;123(4):1051–3.
8. Shander A, Javidroozi M, Naqvi S, Aregbeyen O, Caylan M, Demir S, et al. An update on mortality and morbidity in patients with very low postoperative hemoglobin levels who decline blood transfusion (CME). Transfusion. 2014;54(10 Pt 2):2688–95.
9. Shander A, Javidroozi M, Lobel G. Patient blood management in the intensive care unit. Transfus Med Rev. 2017;31(4):264–71.
10. Shander A, Goodnough LT. From tolerating anemia to treating anemia. Ann Intern Med. 2019;170(2):125–6.
11. Khan F, Singh K, Friedman MT. Artificial blood: the history and current perspectives of blood substitutes. Discoveries (Craiova). 2020;8(1):e104.
12. Speers MA. Providing patients with critical or life-threatening illnesses access to experimental drug therapy: a guide to clinical trials and the US FDA expanded access program. Pharmaceut Med. 2019;33(2):89–98.
13. Van Norman GA. Expanding patient access to investigational new drugs: overview of intermediate and widespread treatment investigational new drugs, and emergency authorization in public health emergencies. JACC Basic Transl Sci. 2018;3(3):403–14.
14. Zumberg M, Gorlin J, Griffiths EA, Schwartz G, Fletcher BS, Walsh K, et al. A case study of 10 patients administered HBOC-201 in

high doses over a prolonged period: outcomes during severe anemia when transfusion is not an option. Transfusion. 2020;60(5):932–9.
15. Agarwal R, Saltz LB. Understanding the right to try act. Clin Cancer Res. 2020;26(2):340–3.
16. Folkers KM, Bateman-House A. Improving expanded access in the United States: the role of the institutional review board. Ther Innov Regul Sci. 2018;52(3):285–93.
17. Donahue LL, Shapira I, Shander A, Kolitz J, Allen S, Greenburg G. Management of acute anemia in a Jehovah's witness patient with acute lymphoblastic leukemia with polymerized bovine hemoglobin-based oxygen carrier: a case report and review of literature. Transfusion. 2010;50(7):1561–7.
18. Jahr JS, Guinn NR, Lowery DR, Shore-Lesserson L, Shander A. Blood substitutes and oxygen therapeutics: a review. Anesth Analg. 2021;132(1):119–29.
19. Guinn NR, Resar LMS, Frank SM. Perioperative management of patients for whom transfusion is not an option. Anesthesiology. 2021;134(6):939–48.
20. Weiskopf RB, Beliaev AM, Shander A, Guinn NR, Cap AP, Ness PM, et al. Addressing the unmet need of life-threatening anemia with hemoglobin-based oxygen carriers. Transfusion. 2017;57(1):207–14.
21. Bachert SE, Dogra P, Boral LI. Alternatives to transfusion. Am J Clin Pathol. 2020;153(3):287–93.
22. Misra H, Bainbridge J, Berryman J, Abuchowski A, Galvez KM, Uribe LF, et al. A phase Ib open label, randomized, safety study of SANGUINATE in patients with sickle cell anemia. Rev Bras Hematol Hemoter. 2017;39(1):20–7.
23. Mackenzie CF, Moon-Massat PF, Shander A, Javidroozi M, Greenburg AG. When blood is not an option: factors affecting survival after the use of a hemoglobin-based oxygen carrier in 54 patients with life-threatening anemia. Anesth Analg. 2010;110(3):685–93.
24. Fitzgerald MC, Chan JY, Ross AW, Liew SM, Butt WW, Baguley D, et al. A synthetic haemoglobin-based oxygen carrier and the reversal of cardiac hypoxia secondary to severe anaemia following trauma. Med J Aust. 2011;194(9):471–3.
25. Posluszny JA, Napolitano LM. Hemoglobin-based oxygen carrier for traumatic hemorrhagic shock treatment in a Jehovah's witness. Arch Trauma Res. 2016;5(2):e30610.
26. Jordan SD, Alexander E. Bovine hemoglobin: a nontraditional approach to the management of acute anemia in a Jehovah's witness patient with autoimmune hemolytic anemia. J Pharm Pract. 2013;26(3):257–60.
27. Lundy JB, Lewis CJ, Cancio LC, Cap AP. Experience with the use of Hemopure in the care of a massively burned adult. Int J Burns Trauma. 2014;4(1):45–8.
28. Thenuwara K, Thomas J, Ibsen M, Ituk U, Choi K, Nickel E, et al. Use of hyperbaric oxygen therapy and PEGylated carboxyhemoglobin bovine in a Jehovah's witness with life-threatening anemia following postpartum hemorrhage. Int J Obstet Anesth. 2017;29:73–80.
29. Sam C, Desai P, Laber D, Patel A, Visweshwar N, Jaglal M. Pegylated bovine carboxyhaemoglobin utilisation in a thrombotic thrombocytopenic purpura patient. Transfus Med. 2017;27(4):300–2.
30. Davis JM, El-Haj N, Shah NN, Schwartz G, Block M, Wall J, et al. Use of the blood substitute HBOC-201 in critically ill patients during sickle crisis: a three-case series. Transfusion. 2018;58(1):132–7.
31. DeSimone RA, Berlin DA, Avecilla ST, Goss CA. Investigational use of PEGylated carboxyhemoglobin bovine in a Jehovah's witness with hemorrhagic shock. Transfusion. 2018;58(10):2297–300.
32. Unnikrishnan A, Pelletier JPR, Bari S, Zumberg M, Shahmohamadi A, Spiess BD, et al. Anti-N and anti-Do(a) immunoglobulin G alloantibody-mediated delayed hemolytic transfusion reaction with hyperhemolysis in sickle cell disease treated with eculizumab and HBOC-201: case report and review of the literature. Transfusion. 2019;59(6):1907–10.
33. Brotman I, Kocher M, McHugh S. Bovine hemoglobin-based oxygen carrier treatment in a severely anemic Jehovah's witness patient after cystoprostatectomy and nephrectomy: a case report. A A Pract. 2019;12(7):243–5.
34. Holzner ML, DeMaria S, Haydel B, Smith N, Flaherty D, Florman S. Pegylated bovine carboxyhemoglobin (SANGUINATE) in a Jehovah's witness undergoing liver transplant: a case report. Transplant Proc. 2018;50(10):4012–4.
35. McConachie S, Wahby K, Almadrahi Z, Wilhelm S. Early experiences with PEGylated carboxyhemoglobin bovine in anemic Jehovah's witnesses: a case series and review of the literature. J Pharm Pract. 2020;33(3):372–7.
36. Mackenzie CF, Dube GP, Pitman A, Zafirelis M. Users guide to pitfalls and lessons learned about HBOC-201 during clinical trials, expanded access, and clinical use in 1,701 patients. Shock. 2019;52(1S Suppl 1):92–9.
37. Romito BT, McBroom MM, Bryant D, Gamez J, Merchant A, Hill SE. The effect of SANGUINATE((R)) (PEGylated carboxyhemoglobin bovine) on cardiopulmonary bypass functionality using a bovine whole blood model of normovolemic hemodilution. Perfusion. 2020;35(1):19–25.
38. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA. 2008;299(19):2304–12.
39. Fergusson DA, McIntyre L. The future of clinical trials evaluating blood substitutes. JAMA. 2008;299(19):2324–6.
40. Shander A, Javidroozi M, Thompson G. Hemoglobin-based blood substitutes and risk of myocardial infarction and death. JAMA. 2008;300(11):1296–7.
41. Norvell DC. Study types and bias-don't judge a study by the abstract's conclusion alone. Evid Based Spine Care J. 2010;1(2):7–10.
42. Carson JL, Noveck H, Berlin JA, Gould SA. Mortality and morbidity in patients with very low postoperative Hb levels who decline blood transfusion. Transfusion. 2002;42(7):812–8.
43. Langhi DM, Covas DT, Marques JFC, Mendrone A, Ubiali EMA, Santis GC, et al. Guidelines on transfusion of red blood cells: prognosis of patients who decline blood transfusions. Hematol Transfus Cell Ther. 2018;40(4):377–81.
44. Shander A, Corwin HL. A narrative review on hospital-acquired anemia: keeping blood where it belongs. Transfus Med Rev. 2020;34(3):195–9.
45. Palaia I, Caruso G, Di DV, Perniola G, Ferrazza G, Panzini E, et al. Peri-operative blood management of Jehovah's Witnesses undergoing cytoreductive surgery for advanced ovarian cancer. Blood Transfus. 2022;20:112–9.
46. Frank SM, Chaturvedi S, Goel R, Resar LMS. Approaches to bloodless surgery for oncology patients. Hematol Oncol Clin North Am. 2019;33(5):857–71.

43 Regulatory Perspectives on Clinical Trials for Oxygen Therapeutics When Transfusion of Red Blood Cells is Not an Option

Toby A. Silverman

Status of Red Blood Cell Transfusion in the United States

In the United States, there are mounting challenges to the provision of an adequate blood supply for current and future patients. These challenges relate to aging of the current donor pool and the need for continuous recruitment of blood donors including those newly eligible to donate; ensuring the adequacy of the blood supply in the case of public health emergencies such as emerging infectious diseases or radiation/chemical agent threats; improvements to the cost and reimbursement model for blood and blood products; the lack of an integrated data system to monitor blood collection and utilizations as well as donor and patient safety; and the need for measures to promote safety and innovation such as the development, use, or implementation of new technologies, processes, and procedures to improve the safety and reliability of the blood supply [1]. Weiskopf et al. have recently summarized these issues related to adequacy of the blood supply, highlighting some issues that have emerged during the SARS-CoV-2 pandemic and pointing again to the recognized need for innovation in the development of safe and effective alternatives to traditional transfusion blood products [2]. Scientific and regulatory considerations on the development of HBOCs for use in lieu of red blood cell transfusion when red blood cells are available and can be used have been discussed elsewhere [3]. Severe anemia when red blood cell transfusion is indicated but is not an option is a setting where a novel product would be of great use. Severe anemia meeting this requirement, outside of a wide-spread blood-borne infection or a mass casualty event, is a rare condition; and the best information about the risk of morbidity and mortality has been collected among patients who have refused transfusion [2, 5].

T. A. Silverman (✉)
Tunnell Government Services, Biomedical Advanced Research and Development Authority (BARDA), Department of Health and Human Services, Washington, DC, USA

At present, there are no available oxygen therapeutic agents for use in humans in the United States [6]. The development of oxygen therapeutics for clinical use therefore occurs in the context of a regulatory environment for approval of drugs and biologics which is briefly summarized in the next section. These requirements should further be understood in the context of the complicated history and standards of review of efficacy performed under the Drug Efficacy Study Implementation program summarized by Weiskopf, et al. [4], the resulting unique standards applied since that time to the approval of blood and blood components, and the differences between the regulation of manufactured products as compared with the regulation of blood and blood components. This chapter will discuss the standards for and special considerations for approval of oxygen therapeutics for use as (manufactured) drugs; it will not discuss the different regulatory considerations that would apply for use of oxygen therapeutics solely under the device regulations. FDA regulations confer great discretionary latitude to agency reviewers to interpret what is required to demonstrate an adequate benefit to risk ratio as discussed below.

General Regulatory Background in the United States

Requirements for Approval/Licensure of Drugs and Biologics

In the United States, new drugs and biologics must be approved before they may be marketed. Drugs are done so under authority of Section 505 of the Federal Food, Drug, and Cosmetic Act (the Act)(United States Code, Title 21, Chapter 9. Section 505 of the Act (21 U.S.C. §355) describes the legal framework for the evaluation, review, and approval of new drugs for introduction into interstate commerce. Biological products are approved under authority of section 351 of the Public Health Service Act (PHS

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_43

Act) (42 U.S.C. §262). The requirements for licensure include a showing that the product is 'safe, pure, and potent.' The history of regulations under these two provisions has been reviewed previously [3]. The most important regulatory concept relates to what constitutes "substantial evidence of effectiveness."

'Substantial evidence' is defined in section 505(d) of the Act as 'evidence consisting of adequate and well-controlled investigations, including clinical investigations, by experts qualified by scientific training and experience to evaluate the effectiveness of the drug involved, on the basis of which it could fairly and responsibly be concluded that the drug will have the effect it purports or is represented to have under the conditions of use prescribed, recommended, or suggested in the labeling or proposed labeling thereof.' (The experts referred to in the law are the medical review staff at FDA). What constitutes "substantial evidence of effectiveness" has long been controversial, particularly as the term relates to rare diseases or to diseases that are serious and or life-threatening for which there is an unmet medical need.

Any clinical trial in support of an NDA or BLA designed to meet the standard of demonstrating 'substantial evidence of effectiveness' must also meet the requirements for being 'adequate and well controlled' as defined in the Code of Federal Regulations at 21 CFR 314.126. What constitutes an adequate control in the case of rare or life-threatening diseases for which there is an unmet medical need, to support a determination of substantial evidence of effectiveness, is discussed below.

In 2019, FDA updated its guidance on providing clinical evidence of effectiveness for human drug and biological products to include discussion of types of controls that may be accepted by the agency [7]. Specifically, the document addresses the issue of adequacy of 'confirmatory evidence' in the case of presentation of data from a single adequate and well-controlled clinical trial, noting that confirmatory evidence could include …'certain types of real world evidence such as …well-documented natural history of the disease.' The wording of the guidance indicates significant discretion on the part of FDA reviewers as to the criteria for determining substantial evidence of effectiveness, an important point for development of any product for a rare or life-threatening condition.

For safety purposes, clinical trials capture information about new and/or novel adverse events, as well as quantitative and/ or qualitative increases in expected adverse events above their underlying background rate/intensity. Studies may also be designed to capture interactions of study product with a wide variety of co-morbid conditions. As noted above, for any indication, a drug or biologic must have a favorable benefit to risk profile, which is a subjective determination.

Expedited Programs

As noted, the drug/biologic development pathway outlined above raises several concerns related to evaluation of products for serious or life-threatening diseases for which there is an unmet medical need. FDA had long had programs to expedite development of drugs and biologics to treat such diseases or conditions, captured in the regulations for expedited programs [8]. The provisions for expedited programs have been modified a number of times and FDA has accordingly issued new/updated guidance (extended expiration date of 04/30/2021) [9]. The four FDA programs outlined in the guidance are intended to facilitate and expedite development and review of new drugs to address unmet medical need in the treatment of a serious or life-threatening condition and include fast track designation, breakthrough therapy designation, accelerated approval, and priority review designation. The threshold conditions that must be met by all development programs under this guidance include a determination that the condition is serious, the drug/biologic is intended to treat a serious condition, and that there is an unmet medical need.

The programs differ based on the type and quantity of information needed to support the potential of the drug or biologic to address these various conditions (fast track versus breakthrough designation); and whether the efficacy endpoint(s) of the study measure an effect on irreversible morbidity or mortality (IMM) or symptoms that represent serious consequences of the disease, or on surrogate endpoint(s) or intermediate clinical endpoint(s) (defined as measurement of a therapeutic effect that can be measured earlier than an effect on IMM) that are reasonably likely to predict an effect on irreversible morbidity or mortality or other clinical benefit, taking into account the severity, rarity, or prevalence of the condition and the availability or lack of alternative treatments (priority review versus accelerated approval). The reader is referred to the guidance documents for a fuller explanation of the criteria for each of these programs.

Real World Evidence

Since passage of the twenty-first Century Cures Act (Pub.L. 114–255) in 2016, there has been considerable discussion about the role of "real world evidence" (RWE) to support and inform regulatory decision-making. Section 3022 of the Cures Act required FDA to establish a program to evaluate the potential use of RWE to help to support the approval of a new indication for a drug approved under section 505(c) of the Federal Food, Drug, and Cosmetic Act (FD&C Act) and to help to support or satisfy post-approval study requirements. In December 2018, FDA published its Framework for

FDA's Real-World Evidence Program, providing examples where RWE has informed regulatory decision-making, as well as advantages and pitfalls of such data collection and use (such as lack of randomization, potential for bias, absence of blinding, data reliability and relevance, gaps in data capture, etc.), noting that "use of RWE for efficacy decision-making is relatively new" [10].

Real World Evidence is defined as "the clinical evidence about the usage and potential benefits or risks of a medical product derived from analysis of real-world data (RWD), and real-world data are defined as "data relating to patient health status and/or the delivery of health care routinely collected from a variety of sources." The framework recognizes that RWE may contribute useful and important information including, for example, information from observational studies and patient registries, to data collected from traditional clinical trials that have carefully prescribed criteria for patient selection, patient care, data acquisition and data analysis.

Of importance for the development of hemoglobin-based oxygen therapeutics for rare or life-threatening disorders is the acknowledgment by FDA that it has exercised regulatory discretion in accepting real world evidence to support other drug and biologic product approvals where approval is based on a single-arm interventional trial in circumstances when the effect size is expected to be large and when randomization is unethical or not feasible. The framework notes that "using external controls has limitations including difficulties in reliably selecting a comparable population because of potential changes in medical practice, lack of standardized diagnostic criteria or equivalent outcome measures, and variability in follow-up procedures." FDA has stated its belief that collection of RWD on patients currently receiving other treatments, together with statistical methods such as propensity scoring, may have the potential to improve the quality of the external control data that are used when randomization may not be feasible or ethical, provided there is adequate detail to capture relevant covariates [10].

Rare Diseases

FDA defines an orphan condition as one that affects fewer than 200,000 persons in the United States, or as one that affects more than 200,000 persons in the United States for which there is no reasonable expectation that the cost of developing and making available in the United States a drug for such disease or condition will recovered from sales in the United States of such drug (Public Law 97–414, as amended; Last updated August 2013). For a drug to qualify for orphan designation both the drug and the disease or condition must meet certain criteria specified in the Orphan Drug Act and FDA's implementing regulations at 21 CFR Part 316. Drugs for orphan indications must meet the same "substantial evidence of effectiveness" standard for approval as drugs for more common conditions including a clear statement of the study objectives, design that permits a valid comparison with a control, and elements to reduce bias. As noted earlier, however, FDA has exercised significant discretion as to the kind and quantity of data a sponsor is required to provide for an individual drug.

FDA has issued two guidance documents on rare diseases that are relevant to the development of hemoglobin-based oxygen carriers for use when transfusion of red blood cells is indicated but is not possible [11, 12]. These guidance documents discuss, among other topics, the use of various types of natural history studies that may inform design, execution, and interpretation of "adequate and well controlled" clinical trials for rare diseases or conditions, and the advantages and disadvantages of retrospective and prospective natural history studies as external controls. FDA's comments on types of controls considered appropriate for regulatory submissions in the two guidance documents on rare diseases comport with its stance articulated within the context of the framework for real world evidence and highlights the cautious approach FDA intends to take with regard to data obtained outside of traditional clinical trials.

Animal Rule

An oxygen therapeutic may be used in situations where clinical trials for the indication are not ethically possible. Clinical trials for the hematopoietic sub-syndrome of Acute Radiation Syndrome is an example of such a clinical scenario. The approval of new drugs when human efficacy studies are not ethical and field trials are not feasible is governed by regulations codified in 21 CFR 314.600 through 314.650 for drugs and 21 CFR 601.90 through 601.95 for biological products. FDA has issued guidance on the development of products under the Animal Rule [13]. This guidance outlines considerations for study design and analysis of studies to support approval under the Animal Rule and issues related to the choice of the animal model from which conclusions about likely efficacy in humans and translation of appropriate doses. The regulations are applicable only if human efficacy studies cannot be conducted because such trials are unethical and field trials after an accidental or deliberate exposure are not feasible. The pathway does not apply to drugs that can be approved for the proposed indication "based on efficacy standards described elsewhere in FDA's regulations" and therefore would generally not relate to development of an oxygen therapeutic for use when red blood cell transfusion is indicated but is not possible. Four conditions must be met to support the use of the Animal Rule pathway: (1) the pathophysiologic mechanism of toxicity of the inciting agent is

reasonably well understood; (2) with few exceptions, the effect is demonstrated in more than one animal species expected to react with a response predictive for humans; (3) the animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity; and (4) the data or information on the kinetics and pharmacodynamics of the product or other relevant data or information, in animals and humans, allows selection of an effective dose in humans. A major consideration for the design, conduct, and interpretation of the results of animal studies of severe anemia relate to the requirements for ethical and humane treatment of animals and therefore the euthanasia criteria specified by local animal use committees. It is beyond the scope of this chapter to examine all of the issues related to care and treatment of animals in such experiments.

Regulatory Programs to Permit Product Use Not Part of Drug Development Pathways Leading to Approval

The common theme for programs such as Expanded Access, Right to Try, and Emergency Use Authorization is that patients have a serious condition or in a life-threatening situation, that there is an unmet medical need of some sort, that participation in a clinical trial is not feasible, that the potential benefits of using the product outweigh the potential risks and that the potential risks are not unreasonable in the context of the disease or condition being treated (captured as completion of Phase 1 clinical trials in the case of Right to Try). The programs are primarily intended to address certain exigent circumstances and, in general, would not be expected to provide sufficient evidence to support a submission (NDA or BLA) for regulatory approval for marketing purposes.

Expanded Access

FDA's regulations on expanded access to investigational drugs for treatment use under an investigational new drug application (IND) (21 CFR part 312, subpart I), went into effect on October 13, 2009. FDA issued guidance on Expanded Access, finalized in June, 2016 and updated in October, 2017 [14]. "Expanded access refers to the use of an investigational drug when the primary purpose is to diagnose, monitor, or treat a patient's disease or condition rather than to obtain the kind of information about the drug that is generally derived from clinical trial." The process is intended to facilitate "access to investigational drugs for treatment use for patients with serious or immediately life-threatening diseases or conditions who lack therapeutic alternatives." Patients for whom expanded access drug use is intended must have a serious or immediately life-threatening disease or condition for which there is no comparable or satisfactory alternative therapy to diagnose, monitor, or treat the disease or condition; the potential patient benefit must justify the potential risks of the treatment use; and those potential risks must not be unreasonable in the context of the disease or condition to be treated. Providing the investigational drug for the requested is not supposed to interfere with the initiation, conduct, or completion of clinical investigations that could support marketing approval of the expanded access use or otherwise compromise the potential development of the expanded access use.

Right to Try

The Right to Try Act (Trickett Wendler, Frank Mongiello, Jordan McLinn, and Matthew Bellina Right to Try Act) was signed into law May 30, 2018 [15]. This law is applicable to patients with life-threatening diseases or conditions who have exhausted all approved treatments and who are not able to participate in a clinical trial. The Act permits patients who meet the conditions listed above and who have provided or for whom informed consent has been provided by a legally authorized representative to have access to an eligible investigational drug for which a phase 1 study has been completed and which has not been approved by FDA for any use. The product in question must be under investigation in a clinical trial that is intended to form the primary basis of a claim of effectiveness in support of FDA approval and which is the subject of an active investigational new drug application. Active development of the drug must be ongoing and use of the product must not be the subject of a clinical hold by FDA. This pathway is in addition to access to investigational drugs under the expanded access pathway for which FDA has also issued guidance. Use of a product in individual patients meeting the criteria of the Right to Try Act would generally not be anticipated to provide information to support a regulatory submission for approval. To date, it appears that use under this law has been very limited.

Emergency Use Authorization

Section 564 of the Federal Food, Drug, and Cosmetic (FD&C) Act authorizes the Secretary of Health and Human Services to permit the emergency use of medical products by declaring an emergency or threat that justifies the emergency use. The purpose is to enable access to "unapproved" products and to enable use of approved products for a new, unapproved indication [16].

In January 2017, FDA issued guidance on Emergency Use Authorization of Medical Products and Related

Authorities (expires 08/31/2022) laying out FDA's general recommendations and procedures for authorization of emergency use of certain unapproved medical products or unapproved uses of a previously approved product to enhance national preparedness for public health, military, and domestic emergencies involving chemical, biological, radiological, and nuclear (CBRN) agents, including emerging infectious disease threats such as pandemic influenza [17]. This authority is separate and distinct from the authority to permit investigational uses of the same products under investigational new drug (IND) or investigational device exemption (IDE) regulations, or under Section 564A which provides for a streamlined mechanisms to facilitate preparedness and response activities involving certain FDA-approved MCMs without FDA issuance of an EUA.

In order for an EUA to be issued, the Secretary of Health and Human Services must determine that the conditions for emergency use exist. FDA may issue an EUA to allow an Medical Counter Measure (MCM) to be used in an emergency to diagnose, treat, or prevent serious or life-threatening diseases or conditions caused by a Chemical/Biological/Radiological/Nuclear (CBRN) agent. The issued EUA may also clearly define the conditions under which the product may be used and may set requirements for data reporting. The EUA may be revoked if the public health emergency ends or if the criteria under which the EUA was issued change, including, but not limited to, the scientific justification for its use. Critically, issuance of an EUA is not intended to be interpreted or seen as "approval" of the product, nor is it intended to establish the product as a new standard of care.

Considerations for Use When Red Blood Cells Are Indicated but Are Not Available or Cannot Be Transfused

This document has reviewed some laws and regulations applicable to the development of hemoglobin-based oxygen carriers for use when red blood cell transfusion is indicated but is not possible. As noted above, programs such as Expanded Access, EUA, and Right to Try do not address the regulatory requirements for approval by the FDA even though they may provide temporary access to product. This review highlights the considerable challenges facing developers of HBOCs to conduct clinical trials for licensure in the United States, especially in the situation of severe and life-threatening anemia. The clinical course and the risk for death as a function of nadir hemoglobin concentration ([Hb]) have been examined in at least five retrospective/prospective analyses of outcomes of patients for whom red blood cell transfusion was not an option and recently summarized [5, 18–21]. The results of assessments in (1) postoperative anemia and (2) among mixed populations of both surgical and non-surgical hospitalized patients ("all-comers") resulted in remarkably similar findings within each category about the trajectory of death as nadir [Hb] concentration decreases. In particular, a recent report by Guinn et al. summarizing prospectively collected data from 271 patients with untransfused severe anemia confirms the results of earlier studies showing that the risk for death increases as a function of decreasing Hb and indicates that time to death decreases with decreasing [Hb] [21]. The remarkable similarity of data within each of the two categories cited above from these sources would appear to fulfill the criterion of "well-documented … course that can be objectively measured and verified, such as high and temporally predictable mortality." Severe untreated anemia is a rare condition that merits consideration of (1) the use of Real-World Evidence as captured in the retrospective/prospective and prospective natural history studies noted above and (2) the use of such studies as external controls for a single arm evaluation of an HBOC to improve survival. Statistical analyses of the data described above have shown that an HBOC improves survival when administered to a severely anemic patient for whom red blood cell transfusion is not an option [2, 4]. The existing evidence suggests that the use of an HBOC in patients with severe anemia is more effective than current standard of care. This would enable a single-arm study against historical data that would obviate the need to consider whether or not to permit the control patients to have access to the HBOC and under what circumstances such access might be permitted, thus making moot the ethical and analytical dilemma such a design entail.

Key Points

- Challenges to adequacy of the blood supply highlight the need for innovation in the development of safe and effective alternatives to traditional transfusion blood products
- Because there are no available oxygen therapeutic agents for use in humans in the United States, the development of oxygen therapeutics for clinical use occurs in the context of a regulatory environment for approval of drugs and biologics.
- In the United States, the statutory standard for approval of a drug or biologic is "substantial evidence of effectiveness" which is determined through the conduct of "adequate and well controlled clinical trials."
- FDA has long had programs to expedite development of drugs and biologics to treat serious or life-threatening diseases or conditions leading to priority review and accelerated approval for marketing purposes.
- FDA has long had programs for making drug/biologic available to patients in need such as Expanded Access programs and Right to Try. Emergency Use Authorization allows temporary availability to products to address

declared health emergency situations. Not all such programs are compatible with requirements for approval.

- For rare diseases, certain aspects of Real-World Evidence may be accepted by FDA to support drug/biologic development but FDA has identified significant issues with the use of Real-World Data to provide external controls for single-arm interventional studies due to the potential for bias. Discussion of the use of Real-World Evidence as external controls requires discussion with FDA before conducting any study with regulatory intent.

Disclaimer The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the U.S. Department of Health and Human Services.

References

1. Klein HG, Hrouda JC, Epstein JS. Crisis in the sustainability of the US blood system. N Engl J Med. 2017;377:1485–8. https://doi.org/10.1093/biolre/ioac031.
2. Weiskopf RB, Glassberg E, Guinn NR, James MFM, Ness PM, Pusateri AE. The need for an artificial oxygen carrier for disasters and pandemics, including COVID-19. Transfusion. 2020;60:3039–45. https://doi.org/10.1111/trf.16122.
3. Silverman TA, Aebersold PM, Landow L, and Lindsey K. Chapter 3. regulatory perspectives on clinical trials for oxygen therapeutics in trauma and transfusion practice blood substitutes Amsterdam ; Boston : Elsevier Academic Press, 2006. London, U.K. and Burlington, MA, USA Robert M. Winslow, MD Editor ISBN-13: 978–0127597607. ISBN-10: 0127597603.
4. Weiskopf RB. The efficacy and safety of liquid stored blood and storage duration: a confused subject; are patients confused? Anest Anal. 2014;119(2):224–9. https://doi.org/10.1213/ANE.0000000000000296.
5. Weiskopf RB, Beliaev AM, Shander A, et al. Addressing the unmet need of life-threatening anemia with hemoglobin-based oxygen carriers. Transfusion. 2017;57:207–14.
6. Jahr JS, Guinn NR, Lowery DR, Shore-Lesserson L, Shander A. Blood substitutes and oxygen therapeutics: a review. Anesth Analg. 2021;132(1):119–29. https://doi.org/10.1213/ANE.0000000000003957.
7. Demonstrating substantial evidence of effectiveness for human drug and biological products guidance for industry. Draft Guidance. https://www.fda.gov/media/133660/download and https://www.fda.gov/vaccines-blood-biologics/guidance-compliance-regulatory-information-biologics accessed 2021-02-07.
8. Food and Drug Administration, Interim Rule, Investigational New Drug, Antibiotic, and Biological Drug Product Regulations; Procedures for Drugs Intended to Treat Life-Threatening and Severely Debilitating Illnesses (53 FR 41516, October 21, 1988).
9. Guidance for Industry Expedited Programs for Serious Conditions – Drugs and Biologics https://www.fda.gov/media/86377/download and http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm. Accessed 2021-02-07.
10. Framework for FDA's real¬world evidence program. December, 2018 https://www.fda.gov/media/120060/download accessed 2021-02-07.
11. Rare Diseases: Common issues in drug development guidance for industry draft guidance. https://www.fda.gov/media/119757/download accessed 2021-02-07, DOI: https://doi.org/10.1155/2020/8364502.
12. Rare Diseases: Natural history studies for drug development guidance for industry. Draft Guidance. https://www.fda.gov/media/122425/download accessed 2021-02-07, DOI: https://doi.org/10.1155/2020/8364502.
13. Product Development Under the Animal Rule Guidance for Industry. Final Guidance. https://www.fda.gov/media/88625/download accessed 2021-02-07.
14. Expanded access to investigational drugs for treatment use — questions and answers. Guidance for Industry. https://www.fda.gov/media/85675/download accessed 2021-02-07.
15. https://www.fda.gov/patients/learn-about-expanded-access-and-other-treatment-options/right-try.
16. https://www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization#euaguidance. Accessed 2021-02-14.
17. Emergency use authorization of medical products and related authorities guidance for industry and other stakeholder. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/emergency-use-authorization-medical-products-and-related-authorities accessed 2021-02-14.
18. Shander A, Javodroozi M, Naqvi S, et al. An update on mortality and morbidity in patients with very low postoperative hemoglobin levels who decline blood transfusion (CME). Transfusion. 2014;54:2688–95. https://doi.org/10.1111/trf.12565.
19. Carson JL, Noveck H, Berlin JA, Gould SA. Mortality and morbidity in patients with very low postoperative Hb levels who decline blood transfusion (CME). Transfusion. 42:812–8. https://doi.org/10.1046/j.1537-2995.2002.00123.x.
20. Beliaev AM, Marshall RJ, Gordon M, Smith W, Windsor JA. Clinical benefits and cost-effectiveness of allogeneic red-blood-cell transfusion in severe symptomatic anemia. Vox Sang. 2012;103:18–24. https://doi.org/10.1111/j.1423-0410.2011.01573.x.
21. Guinn NR, Cooter ML, Weiskopf R. Lower hemoglobin concentration decreases time to death in severely anemic patients for whom blood transfusion is not an option. J Trauma Acute Care Surg. 2020:803–8. https://doi.org/10.1097/TA.0000000000002632.

Index

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3