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Editors

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*To my parents and Hanna, Bryan, Martha and Edward whose love and support made it all possible*

—Hae Won Kim

*To my wife, Reva, whose ever-present love and support has made it possible for me to pursue my academic interests and passions*

—A. Gerson Greenburg

# Preface

It has been a while since a comprehensive review of the status of Hemoglobin-Based Oxygen Carriers (HBOCs) has been assembled. Indeed, the field in many ways has become orphaned as clinical and technologic advances in blood banking and the management of anemia move forward. Spurred on in the early 1980s with the onset of AIDS, HBOC development was rich and productive, an alternative to risky red cell transfusions. This led to a more modern approach to red cell transfusion and HBOCs were more or less relegated to specific applications as "oxygen therapeutics". All the while of course, there continued a need for an oxygen therapeutic if only for use when blood was neither an option nor available. Clinical trials with a number of HBOCs went forward and all failed to produce results sufficient to permit FDA regulatory approval. In the early part of the current century it appeared as if there was no need for such a product and the regulatory environment continued to be challenging.

We decided to bring the field up-to-date with the construction of this book. Dedicated to HBOCs alone, our intention was to bring together in one place the current status of the field, a starting point for those interested in joining in on the adventure of discovery, a place for the veterans of the fight to read and reflect on what was and thinking about creating "what can be". We hold that there continues to be a need for an oxygen therapeutic and that need can be met successfully with a HBOC. In fact, there is an urgency to develop HBOCs as an alternatives to donor blood for transfusion. Based on current world demographics, it has been predicted that, in coming decades, there would be a serious shortage of transfusable blood because of higher demands due to rapidly aging populations in many countries while relative decrease in eligible younger donors.

The organization of this work is rather traditional, starting with an historical overview and statement of scientific principles as the foundation for the products. Moving onto a specific application in hemorrhagic shock is a natural development as in addition to AIDS, it was and remains a clearly stated potential application. A discussion of some of the current issues and regulatory framework rounds out the establishment of a foundation for the rest of the book.

A rather large section discussing many of the newer and older approaches to HBOC development follows. Here, the many different ideas for resolving some of the questions posed by regulatory and experiential reports are addressed creating a plethora of new modifications and processes as they move along. Clearly, the next

step is to identify and catalog the potential applications for these products. While the universe of potential applications exceeds those presented here, these are but a few specifics linked to unique HBOCs. There are many more possible, a near endless list could be generated.

There follows a section on preclinical evaluation, a critical step in the regulatory process. Here again, generalizations from specific applications and products form the reference model. In the background remains the issue of how appropriate and useful are animal models in the development of human-use products and we are aware of many HBOC preclinical studies that may not be relevant to how humans will respond to the product given the variation in human physiology and disease state.

As the failure of clinical trials has been the major blockade to regulatory approval it seems appropriate to include commentary on this issue. There are critical questions posed in these writings that should be considered when contemplating HBOC clinical trials. As a corollary to a discussion of clinical trials, a section on the adverse events so critical to gaining an understanding of how they come about and more importantly how they can be mediated or prevented is a logical follow-on.

We think this discussion is critical to understanding how HBOCs work, how their use to date in general, and in trials specifically as well as in compassionate use situations. There are many reasons for an adverse event to appear and it is not always clear in the rigid analysis of data from a randomized trial that the HBOC is responsible for the observation.

To that end, we conclude with a white paper that proposes a consortial approach to facilitate development of safer and more effective HBOCs. A HBOC research consortium composed of committed independent investigators would allow objective investigations of critical issues based on unbiased scientific principles and methods.

This book is meant to bring an up-to-date perspective to the HBOC field, a reference for where the field stands at this point in time. It is our sincere hope that it accomplishes this objective and meets your specific needs.

Sincerely,

October 2013

Hae Won Kim
A. Gerson Greenburg

# Acknowledgments

We are deeply grateful to all the Authors who, despite other priorities that demand on their time, have willingly and enthusiastically contributed to our book. Most of them are longtime colleagues who have dedicated a significant portion of their careers to HBOC research and development. Writing a book chapter is regarded by many as secondary and a distraction, diverting energy from discovery and creativity. We hope that this finished product serves as a small consolation for their efforts as it provides fresh insights for the development of safer and more effective HBOCs. Lastly, HWK would like to express a special thanks to Professor Chi-Ming Hai and Professor Chair Wayne Bowen, Department of MPPB program at Brown University, who have kindly made arrangements that allowed him to work on this worthy task.

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# Part I
# Introduction and Scientific Principles

# Chapter 1
# From Hemoglobin Based Oxygen Carrier to Oxygen Therapeutics, Blood Substitutes, Nanomedicine and Artificial Cells

**Thomas Ming Swi Chang**

## 1.1 Introduction

Hemoglobin (Hb), a tetrameric protein, is responsible for the transport of oxygen in Red Blood Cells (RBC) (Perutz 1980). Attempts to use hemolysate (Amberson 1937) and stroma-free Hb as Hb based oxygen carrier (Rabiner et al. 1967) resulted in nephrotoxicity and cardiovascular adverse effects (Savitsky et al. 1978). Thus, nanobiotechnological modification is needed before it can be used (Chang 1964)

### *1.1.1 Artificial Red Blood Cell*

The first artificial RBC that contains Hb and RBC enzymes (Fig. 1.1) have oxygen dissociation curve similar to RBCs, since 2-3-DPG is retained inside (Chang 1957, 1964). Hb stays inside as tetramers and RBC enzymes like carbonic anhydrase and catalase retain their activities (Chang 1964, 1972). These artificial RBCs do not have blood group antigens on the membrane and therefore do no aggregation in the presence of blood group antibodies (Chang 1972). However, the single major problem was the rapid removal of these artificial cells from the circulation. Nanobiotechnology based soluble complex was therefore investigated to increase the circulation time.

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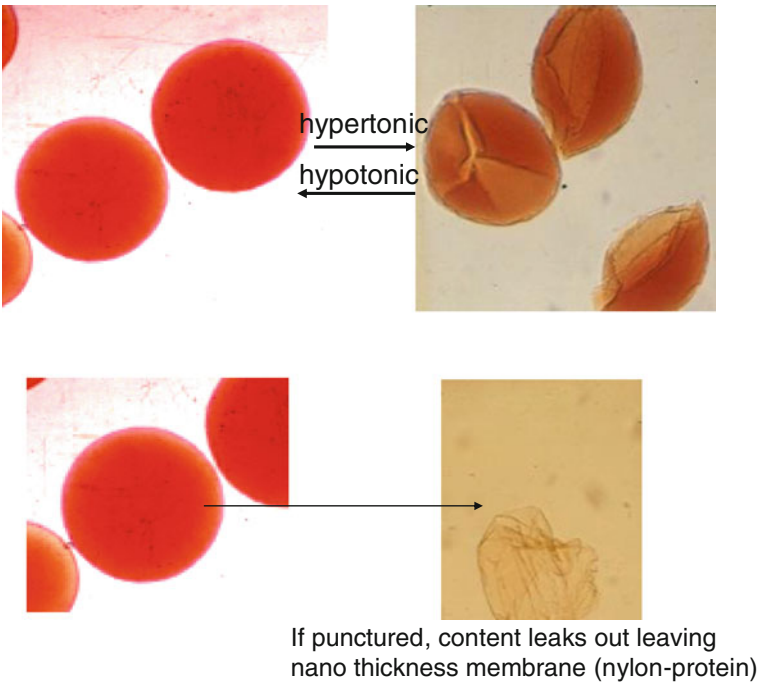


**Fig. 1.1** Micro dimension artificial RBC with ultrathin nylon-protein membrane from Chang 1965

### *1.1.2 First Generation Hemoglobin Based Oxygen Carriers*

The first nanobiotechnology approach reported is the crosslinking of Hb into ultrathin polyhemoglobin (PolyHb) membrane with nanodimension thickness (Chang 1964, 1965, 1972) (Fig. 1.2). This is used to form the membrane of artificial RBCs (Chang 1964, 1965, 1972). If the emulsion is made very small, then the whole submicron artificial cells can be crosslinked into PolyHb of nanodimension. Glutaraldehyde can crosslink Hb into soluble PolyHb each consisting of an assembly of 4–5 Hb molecules (Chang 1971) (Fig. 1.2). Crosslinking of Hb into PolyHb includes both intermolecular and intramolecular crosslinking. Bunn and Jandl (1968) chose to do only intramolecular crosslinking of a single Hb molecule. Sebacyl chloride crosslinks hemoglobin and diamine to form polyamide conjugated hemoglobin (Chang 1964, 1972) (Fig. 1.2). An useful extension of this is the crosslinking of single protein molecule to soluble polymers like dextran (Wong 1988) and polyethylene glycol (PEG) (Park et al. 1981) (Fig. 1.2).

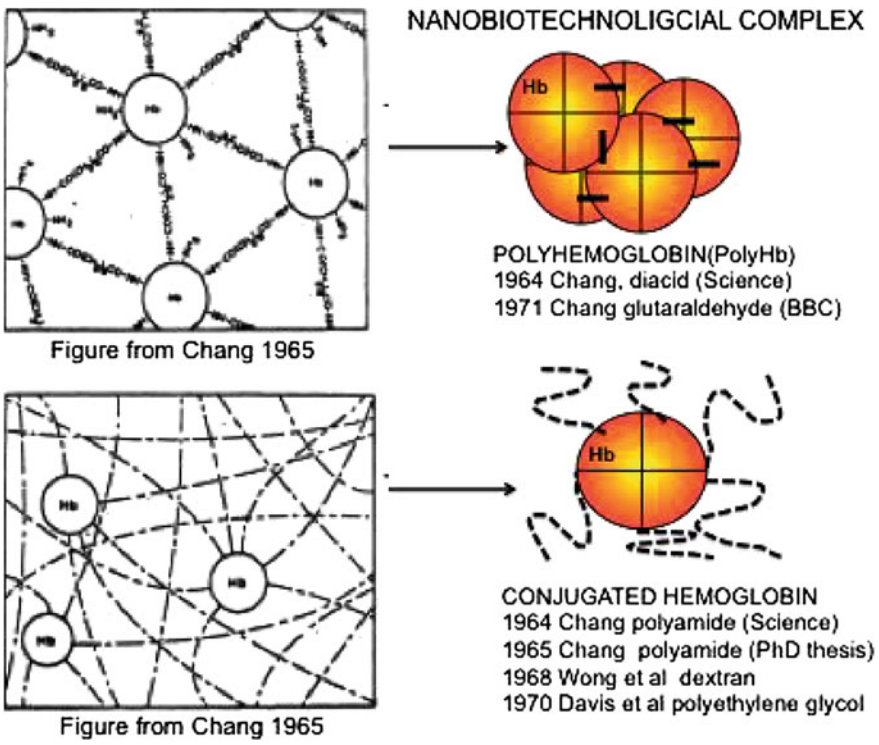


**Fig. 1.2** *Upper left* basic method of using bifunctional agents to assemble and crosslink Hb into PolyHb. *Upper right* soluble complex of PolyHb. *Lower left* basic method of conjugating Hb to polymer. *Lower right* conjugation of Hb to polyamide, soluble dextran or PEG (with copyright permission from Chang 2007 Monograph on Artificial Cells)

### *1.1.3 Little Interest Until It was Too Late When HIV Contaminated Donor Blood Arrived*

It was thought by many that blood substitute was a simple matter to develop without the need for basic research. Thus, there was little or no interest in blood substitutes at that time. As a result, researchers had to channel their major research effort into other areas (Chang 1964, 1972, 2005, 2007, 2009–2013). It was only when the HIV contaminated donor blood crisis came in the 1980s that intense interest started to focus on blood substitutes. Concerned researchers and industries then placed maximal effort into solving this urgent need. Unfortunately, without the needed basic research and basic knowledge, development of blood substitutes had to be done by trial and error. Furthermore, the urgent need was such that the simplest and shortest route was chosen, in the form of simple oxygen carriers like Hb based oxygen carrier.

## 1.2 First Generation Hemoglobin Based Oxygen Carriers

### *1.2.1 Four Common Types*

The four types of most commonly studied first generation Hb based oxygen carriers are shown in Figs. 1.3 and 1.4. These are PolyHb, conjugated Hb, intramolecularly crosslinked tetrameric Hb and recombinant Hb.

### *1.2.2 PolyHb for Use as Oxygen Carrier for Transfusion*

The 1971 basic principle of glutaraldehyde crosslinked PolyHb (Chang 1971) (Fig. 1.2) has been independently developed most extensively by centers around the world (Dudziak and Bonhard 1980; DeVenuto 1982; Keipert et al. 1982; Keipert and Chang 1987; Segal et al. 1983; Moss et al. 1988; Gould et al. 1995; Pearce and Gawryl 1998). Unlike RBCs, there is no blood group, and thus PolyHb can be given on the spot, without waiting for typing and cross-matching in the hospital. They are also free from infective agents such as HIV, hepatitis C, bacteria, parasites and so on. Whereas donor blood has to be stored at 4 °C and is only good for 42 days, PolyHb can be stored at room temperature for more than one year. Thus, PolyHb can have important uses in a number of clinical conditions.

The following is a summary of the recent ongoing research, development and clinical use.

### *1.2.3 Glutaraldehyde Crosslinked Human PolyHb*

Gould and Moss started the Northfield Laboratory to develop glutaraldehyde crosslinked human PolyHb (Sehgal et al. 1983; Moss et al. 1988; Gould et al. 1995).

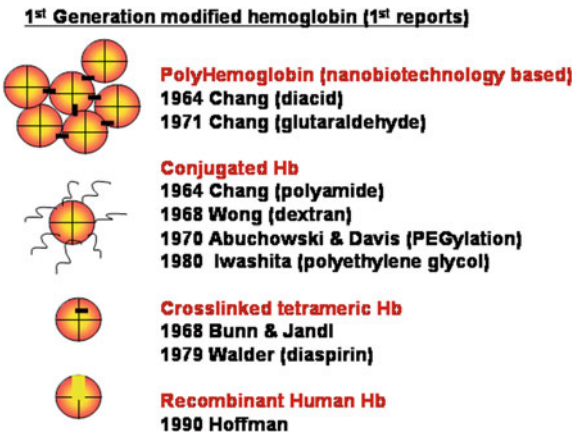


**Fig. 1.3** First generation Hb based oxygen carriers (with copyright permission from Chang 2007 Monograph on Artificial Cells)

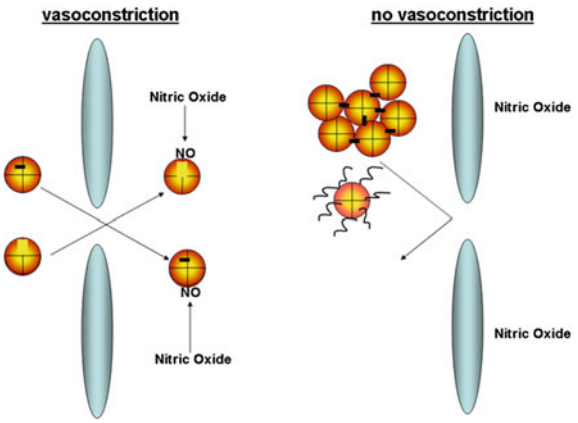


**Fig. 1.4** *Left* Stabilized tetrameric Hb is small enough to cross intercellular junction and remove interstitial nitric oxide resulting in vasoconstriction. However this will not happen if stabilized tetrameric Hb has been modified to prevent NO removal. *Right* PolyHb and conjugated Hb are large enough so they cannot cross the intercellular junction to cause vasoconstriction. Exceptions are in endothelial dysfunction or those preparations that contain substantial amount of tetrameric Hb—unless modified to prevent removal of nitric oxide (with copyright permission from Chang 2007 Monograph on Artificial Cells)

Their clinical trial shows that this can replace blood lost in trauma surgery by keeping the blood Hb at an acceptable level (Gould et al. 2002). This clinical trial on 171 patients showed 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. More recently, they have carried out clinical trials on its used in pre-hospital emergencies since no typing and cross-matching is needed and it can be used right on the spot. This clinical trial involves giving this in the ambulance to about 700 hemorrhagic shock patients shows that PolyHb can maintain the patient for 12 h after reaching the hospital. In the control group, most of the patients need blood transfusion on reaching the hospital (Moore et al. 2009).

### *1.2.4 Glutaraldehyde Crosslinked Bovine PolyHb*

Bing L, Wong and Carl Rausch were the cofounders of Biopure to start work in this. Recent overviews of the development and extensive clinical trials are available (Pearce and Gawryl 1998; Jahr et al. 2008; Greenburg et al. 2008; Greenburg 2013). For example, they have carried out multicenter, multinational, randomized, single-blind, RBC-controlled Phase III clinical trials in patients undergoing elective orthopedic surgery. 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. Russia and South Africa have approved this for routine clinical uses in patients.

### *1.2.5 Other Sources of Hemoglobin for PolyHb*

In addition to Hb from outdated human donor blood, bovine Hb, as mentioned above is another source (Feola et al. 1983; Wong et al. 2011). Other sources of Hb have also been used for PolyHb. These included, for example, preclinical studies on porcine Hb (Zhu et al. 2007; Zhu and Chen 2013) and Hb from human placental blood (Li et al. 2006). These two groups have carried out extensive laboratory and preclinical studies. Other possible sources of Hb include recombinant Hb (Hoffman et al. 1990), marina Hb (Rousselot et al. 2006) and others.

### *1.2.6 Conjugated Hemoglobin*

In the presence of diamine, sebacyl chloride crosslinks Hb with polyamide to form conjugated Hb (Chang 1964, 1965) (Fig. 1.2). An extension of this is the crosslinking of single Hb molecule to soluble polymers like dextran (Wong 1988; Tam et al. 1976) or PEG (Abuchowski and Es 1977; Iwashita 1992; Yabuki 1990; Shorr, Viau and Abuchowski 1996; Li 2005; Winslow 2006; Liu and Xiu 2008; Seetharama et al. 2013) (Fig. 1.3). PEG-Hb shares many of the advantages of PolyHb as described above. More details are available in other later chapter. Clinical trials are ongoing for two types of PEG-Hb (Winslow 2006; Li 2005; Liu and Xiu 2008). A Double-Blind, Randomized, Multicenter Study of MP4OX has been reported (Van der Linden et al 2011).

### *1.2.7 Stabilized Tetrameric Hb and Other Factors*

In addition to glutaraldehyde crosslinked PolyHb and conjugated Hb there are other ways of modifying Hb (Fig. 1.3). These include intramolecularly crosslinked tetrameric Hb (Walder 1979; Przybelski et al. 1996; Burhop and Estep 2001), recombinant human Hb (Looker 1992; Shoemaker 1994). Some have resulted in adverse effects like vasoconstriction in clinical trials. This has led to the proposal that the intercellular junctions of the endothelial lining of vascular wall allow tetrameric Hb to enter into the interstitial space. There, Hb acts as a sink in binding and removing nitric oxide needed for maintaining the normal tone of smooth muscles. This results in the constriction of blood vessels and other smooth muscles especially those of the esophagus and the GI tract (Fig. 1.4). However, this can be

avoided if nitric oxide removal is prevented by a specially designed recombinant Hb (Doherty et al. 1998) or a modified form of stabilized intramolecularly crosslinked Bovine Hb (Wong and Kwok 2011) or by the administration of nitric oxide (Yu et al. 2010; Zapol 2012).

Even PolyHb, for example raffinose polymerized Hb (Hsia et al. 1992), that contains more than 30 % tetrameric Hb can have some vasoconstriction effects. Those PolyHb or conjugated Hb that contain high levels of uncrosslinked Hb or low molecular weight PolyHb could also have adverse effects (Kim and Greenburg 1997; Chang 1997, 2007; Bucci 2011, 2013) (Fig 1.4). There are also other factors including pathological characteristics of patients, like endothelial dysfunction (Yu et al. 2010). Furthermore, the design of preclinical and clinical study is complicated (Greenburg and Kim 1992; Zuck 1994; Fratantoni 1994; Klein 2000; Chang, 1997, 2007; Winslow 2006; Greenburg 1992, 2008, 2013). As mentioned earlier, vasoconstriction can be avoided if nitric oxide removal is prevented by a specially designed recombinant Hb (Doherty et al. 1998) or a modified form of stabilized intramolecularly crosslinked Bovine Hb (Wong and Kwok 2011) or by the administration of nitric oxide (Yu et al. 2010; Zapol 2012).

In medicine, nothing can be considered to be a "cure all". First generation Hb oxygen carriers are suitable for many clinical conditions especially in patients with no endothelial dysfunction or as discussed below in patients with no sustained ischemia or elevated tissue pCO2. Thus, one cannot attempt to combine the clinical trial results of different types of Hb based blood substitutes and different clinical conditions into a single meta-analysis as has been done (Natanson et al. 2008). First generation Hb based oxygen carrier is like penicillin that is a good basic method for use in many conditions. However, as for penicillin, with experience gained from clinical results, new generations need to be developed to complement and supplement the first generation. However, there is no reason to use a more complicated and more expensive new generation system if the clinical conditions can be treated safely and effective using the first generation ones.

## 1.3 New Generations

### *1.3.1 Nanobiotechnology to Assemble Hemoglobin with Antioxidant Enzmyes*

In heart attack or stroke due to arterial obstruction, RBCs, being 7 micron in diameter, cannot go through the obstruction (Fig. 1.5). PolyHb being in solution, can more readily go through the obstruction to supply the needed oxygen (Fig. 1.5). However, this has to be done early since if there is much delay, PolyHb alone might result in the production of oxygen radicals that can cause tissue injury (ischemia reperfusion injuries). As mentioned above PolyHb is also ideal for use in hemorrhagic shock since it can be given on the spot. Here again there is a window

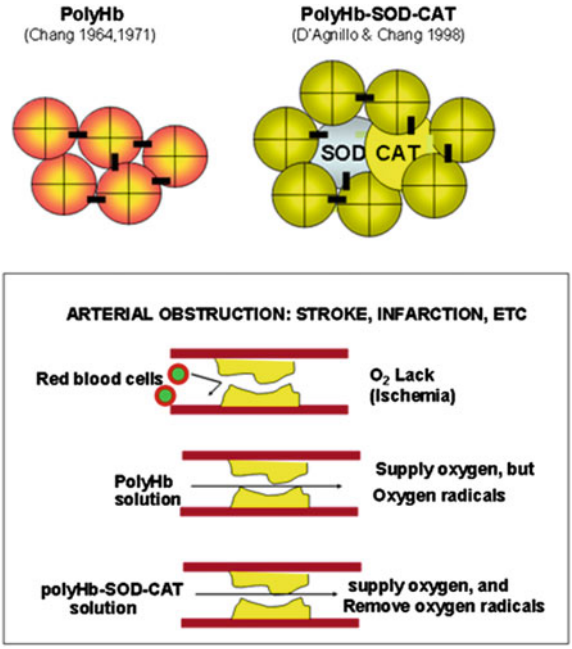


**Fig. 1.5** Arterial obstruction from the narrowing of artery can result in Stroke and heart attack. RBCs being 7–8 micron in diameter have difficulty flowing through obstructed vessels to supply the needed oxygen. PolyHb being a solution can perfuse through to supply the needed oxygen. However, if oxygen lack is prolonged, reperfusion with an oxygen carrier can release damaging oxygen radicals. One possible solution is to use PolyHb-SOD-CAT that has the dual function of an oxygen carrier and the ability to remove oxygen radicals (with copyright permission from Chang 2007 Monograph on Artificial Cells)

of safety since ischemia reperfusion injury can result if there is much delay. Even antioxidant enzymes normally present in RBCs are not enough to prevent this problem in severe sustained hemorrhagic shock (Biro et al. 1995).

We therefore design a soluble nanobiotechnological complex of PolyHb containing two RBC antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) in the form of PolyHb-SOD-CAT (D'Agnillo and Chang 1998) (Figs. 1.5 and 1.6) In this form the SOD and CAT can be enhanced to be much higher than those in RBCs. Thus in a rat stroke model, after 60 min of ischemia, reperfusion with PolyHb resulted in significant increase in the breakdown of the blood–brain barrier and an increase in brain water (brain edema) (Powanda and Chang 2002). On the other hand, PolyHb-SOD-CAT did not result in these adverse changes (Powanda and Chang 2002) (Fig. 1.6). Ischemia–reperfusion injury in severe sustained hemorrhagic shock can result in damage to the intestine with leakage of E-coli or endotoxin to the systemic circulation resulting in irreversible shock. Thus, we tested and found that PolyHb-SOD-CAT can decrease oxygen radicals formation in a rat model of intestinal ischemia reperfusion (Razack et al. 1997). Others have used this for pancreatic beta cells in rats (Nadithe and Bae 2011a); for myocardial infarction

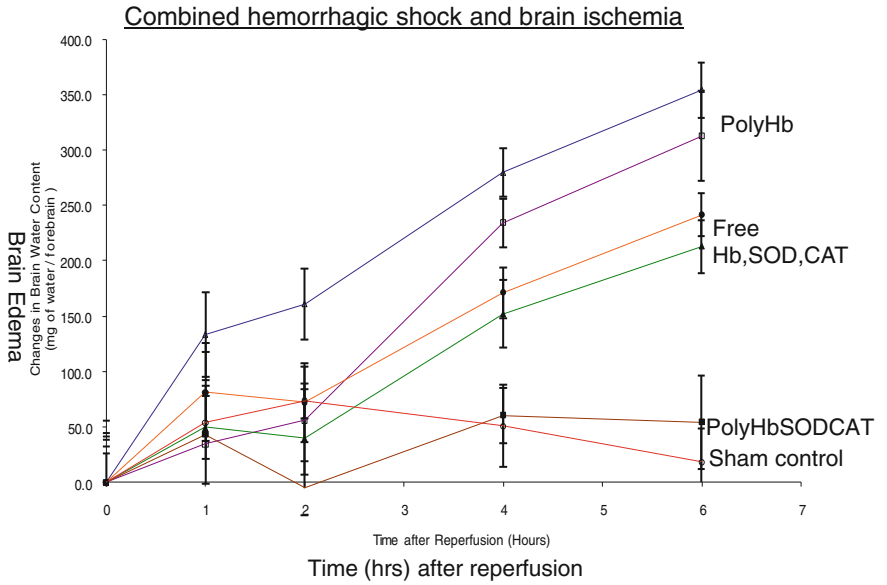


**Fig. 1.6** Different fluids were infused after 90 min of combined hemorrhagic shock and brain ischemia. Brain edema was followed. PolyHb-SOD-CAT treated animals did not have any brain edema. On the other hand, PolyHb or free solution of Hb, SOD and CAT caused significant increases in brain edema (From Powanda and Chang 2002 with copyright permission from J. Artificial Cells, Blood Substtutes and Biotechnology)

attenuation in rats (Wang et al. 2012) and rat kidney transplantation from Korea (Chang et al. 2004). Hsia extended the PolyHb-SOD_CAT approach to prepare a Hb with synthetic antioxidant based on the covalently binding of nitroxides (Buehler et al. 2004; Ma and Hsia 2013). In another approach, using his background on the subject (Alayash 2004), a Hb-haptoglobin complex can also be used to protect against oxidative stress (Jia 2013). Another one is Arenicola marina Hb (Rousselot et al. 2006) with antioxidant activity. Simoni et al. (1997) added a pharmacological solution with antioxidant function to their modified Hb. More details will be available by these authors in later chapters.

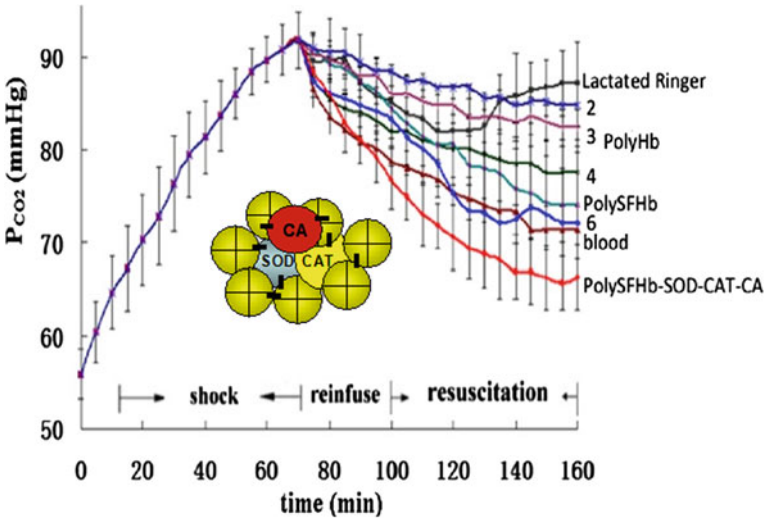


**Fig. 1.7** Hemorrhage shock rats maintained at a mean arterial blood pressure of 30 mm Hg. The tissue pCO2 increases steady with time. Reinfusion of different fluids shows that lactated Ringer salt solution or PolyHb did not lower the tissue pCO2 as much as blood or PolyHb-SOD-CAT-CA. The later is even more effective than blood. (From Bian et al. 2013a with copyright permission from J Artificial Cells, Nanomedicine and Biotechnology)

### *1.3.2 Nanobiotechnology to Assemble Hemoglobin with Superoxide Dismutase, Catalase and Carbonic Anhydrase Resulting in a Carrier for Both Oxygen and Carbon Dioxide with Enhanced Antioxidant Activity*

PolyHb-SOD-CAT is an oxygen carrier with enhanced antioxidant properties. Since RBCs also carry out the important function of transport of carbon dioxide from tissue to the lung for excretion, do we need this component? Sims et al. (2001) used a novel microelectrode to measure tissue pCO2 in animal model of severe hemorrhagic shock. He shows that mortality is related to the elevation of tissue pCO2. The enzyme carbonic anhydrase (CA) in RBC is the major means for the transport of tissue $CO_2$ to the lung. We therefore use the nanobiotechnological method to assemble CA with Hb and antioxidant enzymes to form PolyHb-SOD-CAT-CA (Bian et al. 2011). Our recent study in a rat hemorrhagic shock model shows that this is more efficient than RBC in lowering the elevated pCO2 level in the tissue in a hemorrhagic shock rat model (Bian et al. 2013a) (Fig. 1.7).

### 1.3.3 Stability of PolyHb-Enzymes Nanobiotechnology Complexes

One obvious question is how stable are these type of PolyHb-enzymes complexes. Unlike PolyHb, the enzyme component may not be sufficiently stable with storage especially in room temperature and hot climate. Our very recent study shows that freeze-dried powder preparation of PolyHb-SOD-CAT-CA is stable with very long-term storage at −80 and 4 °C. It is also very stable at room temperature and even at 37 °C when compared to the solution Bian, Yang and Chang (2013). The freeze-dried powder is much easier for storage since it takes up little space also being very light and compact, it is easy for transportation. This is especially important for emergency, disasters or war.

### 1.3.4 Nanobiotechnology to Assemble Hemoglobin and Fibrinogen into an Oxygen Carrier with Platelet-Like Activity

PolyHb can replace the Hb level in very severe hemorrhage, but platelets also need to be replaced (Gould et al. 2002). 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 (Wong and Chang 2007) (Fig. 1.7). We use nanobiotechnology to assmble Hb with fibrinogen to form PolyHb-fibrinogent (Wong and Chang 2007). Using this, we can replace up to 98 % of the total blood volume with PolyHb-fibrinogen without causing clotting problems (Wong and Chang 2007) (Fig. 1.8).

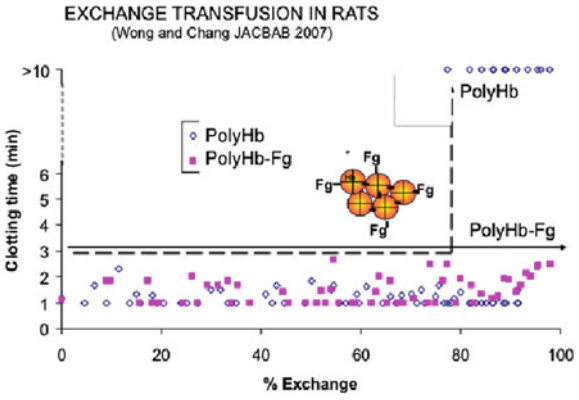


**Fig. 1.8** Exchange transfusion in rats. There is clotting problem when more than 80 % of blood has been exchanged with PolyHb. There is no problem with clotting when 98 % of the blood is replaced with PolyHb-fibrinogen with platelet-like activity (From Wong and Chang, 2007 with copyright permission from J. Artificial Cells, Blood Substitutes and Biotechnology)

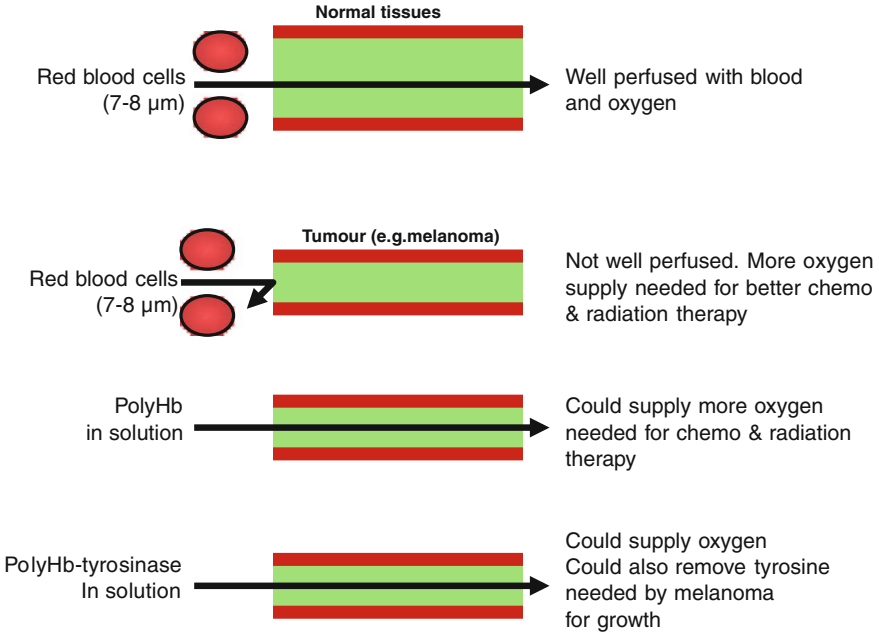


**Fig. 1.9** Unlike RBC, PolyHb and PEG-Hb can better perfused the microcirculation of tumours. This increases the low oxygen tension in tumour and thus increases their sensitivity to radiation and chemotherapy. PolyHb-tyrosinase combine this effect with the removal of tyrosine needed for the growth of melanoma (with copyright permission from Chang 2007 Artificial Cells)

### *1.3.5 Hemoglobin Based Oxygen Carriers in the Treatment of Cancer*

Abnormal microcirculation in tumour leads to decrease in perfusion by oxygen carrying RBCs. PolyHb can more easily perfuse the abnormal microcirculation of tumours to supply oxygen needed for chemotherapy or radiation therapy (Robinson et al. 1995; Teicher 1995) (Fig. 1.9). Thus, PEG conjugated Hb has been used this way (Han et al. 2012; Shorr et al. 1996). PolyHb also decreases the growth of tumour and increases the lifespan in a rat model of gliosarcoma brain tumour (Pearce and Gawryl 1998).

We have crosslinked tyrosinase with Hb to form a soluble PolyHb-tyrosinase complex (Yu and Chang 2004) (Figs. 1.9, 1.10). 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 (Yu and Chang 2004) (Fig. 1.10). Our more recent study include the use of PLA and PEG-PLA membrane nano artificial cells containing polyHb-tyrosinase (Furstier and Chang 2012; Wang and Chang 2012).

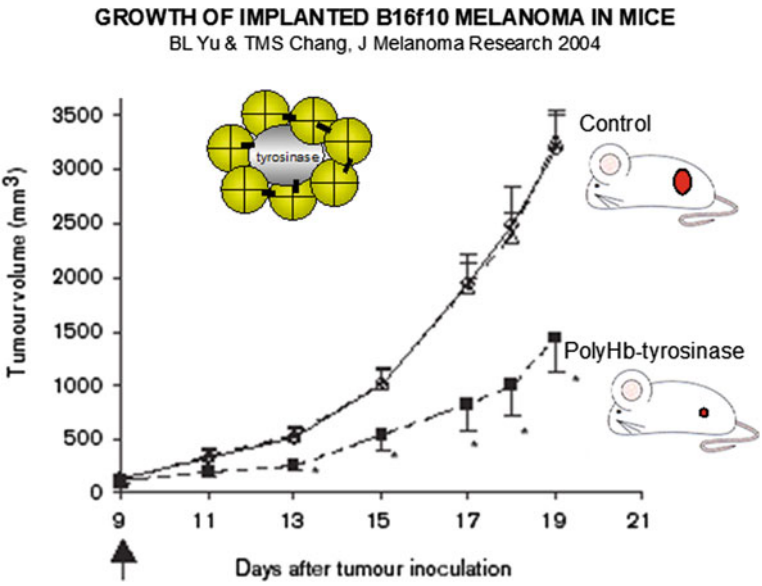


**Fig. 1.10** Effects of daily intravenous injection of PolyHb-tyrosinase on tumor growth of B16F10 melanoma in mice. (i) sham control: no intravenous injection; (ii) saline control: (iii) PolyHb-tyrosinase group (with copyright permission from Chang 2007 Monograph on Artificial Cells)

## 1.4 Nanodimension Complete Artificial Red Blood Cells

### *1.4.1 Early Artificial Red Blood Cells*

The first artificial RBCs (Fig. 1.1) have all the in vitro function of RBCs as shown by oxygen dissociation curve (Chang 1957), carbonic anhydrase activity (Chang 1964) and catalase activities (Chang and Poznansky 1968). These artificial RBCs do not have blood group antigens on the membrane and therefore do no aggregation in the presence of blood group antibodies (Chang 1972). However, the single major problem is the rapid removal of these artificial cells from the circulation. Much of the studies since that time are to improve survival in the circulation by decreasing uptake by the reticuloendothelial system. Since removal of sialic acid from biological RBCs resulted in their rapid removal from the circulation (Chang 1965, 1972), we started to modify the surface properties on artificial RBCs. This included synthetic polymers, negatively charge polymers, crosslinked protein, lipid–protein, lipid–polymer and others (Chang 1965, 1972). As discussed below, artificial RBCs have since been extensively explored by many researchers around the world. These include Beissinger, Bian, Chang, Farmer, Gao, Hunt, Kobayashi, Lee, Mobed, Nishiyia, Rabinovic, Rudolph, Sakai, Schmidt, Sinohara, Szebeni, Takeoka, Tsuchida, Takahashi, Usuba and many others.

### 1.4.2 Bilayer Lipid Membrane Nano Artificial RBC

Bangham (1965) reported the preparation of liposomes each consisting of microspheres of onion like concentric lipid bilayers—multi-lamellar. This was initially used as membrane models in basic research. The large amount of lipid in the multilamellar liposome limits the amount of water-soluble drugs that can be enclosed. Thus, the basic principle and method of preparing artificial cells using ether as the dispersing phase (Chang 1957, 1964) was extended by into what they call an "ether evaporation method" to form single bilayer (unilamellar) lipid membrane liposomes for drug delivery (Deamer and Bangham 1976). Using an extension of this a major progress is the preparation of submicron lipid membrane artificial RBC (Djordjevich and Miller, 1980; Farmer et al. 1988; Philips et al. 1999; Rudolph 1994; Kobayashi et al. 2005; Tsuchida et al. 2006; Sakai 2013). By the addition of PEG to the lipid membrane, the circulation time has been increased to a half time of about 36 h in rats (Philips et al. 1999). These advances make it now possible to scale up for detailed preclinical studies towards clinical trial (Tsuchida 1998; Kobayashi et al. 2005; Sakai 2013). The uptake is mainly by the reticuloendothelial system. It is possible to replace 90 % of the RBCs in rats with these artificial RBCs (Tsuchida 1998; Kobayashi et al. 2005; Sakai 2013). The animals with this percentage of exchange transfusion still remain viable. Studies also reported effectiveness in hemorrhagic shock. There are no changes in the histology of brain, heart, kidneys and lungs of rats. More details will be available in a later chapter by these authors.

### 1.4.3 Nanodimension Biodegradable Polymeric Artificial Cells

Using a modification of this author's earlier method of micron dimension biodegradable polymeric membrane artificial cells (Chang 1976) we have prepared biodegradable polymeric membrane nano dimension PLA artificial RBCs (Chang 1997, 2007; Chang et al. 2003; Yu and Chang 2004). This decreases the amount of lipid needed for the nano artificial cells. Polymer membrane is stronger than

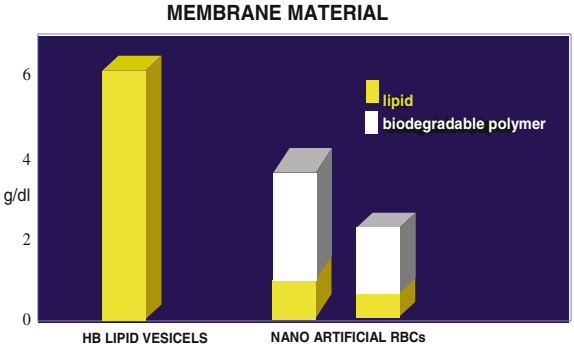


**Fig. 1.11** Amount of membrane material in Hb lipid vesicles compared to PLA nano RBCs (with copyright permission from Chang 2007 Monograph on Artificial Cells

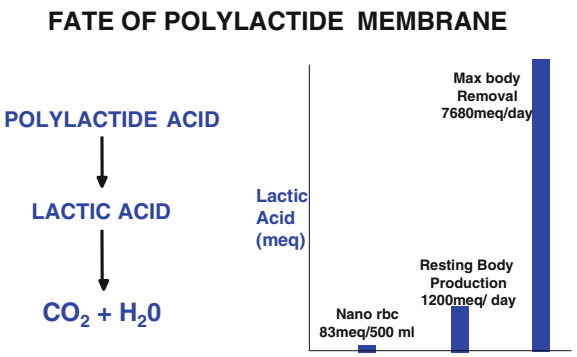


**Fig. 1.12** Polylactide membrane in PLA nano RBCs is biodegraded into lactic acid and finally water and carbon Lactic acid in 500 ml of PLA nano artificial RBC compared to resting body production and maximal body removal capacity (Chang 2007)

bilayer lipid and a thinner polymer membrane can be used. Figure 1.11 shows the amount of membrane material in Hb lipid vesicles compared to PLA nano RBCs.

Polylactide membrane in PLA nano RBCs is biodegradable into lactic acid and finally water and carbon dioxide and thus is not retained in the reticuloendothelial system (Fig. 1.12). Lactic acid in 500 ml of PLA nano artificial RBC is 83 meq/500 ml compared to resting body production of lactic acid of 1,200 meq/day and a maximal body removal capacity for lactic acid of 7,860 meq/day (Fig. 1.12).

These nano artificial RBC of 80–150 nm contain all the RBC enzymes and can convert methemoglobin to Hb (Chang et al. 2003) (Fig. 1.13). The membrane is not permeable to larger molecules, but freely permeable to small molecules like glucose and reducing agents from plasma. In vitro study shows that when incubated at 37 °C MetHb increases quickly (Fig. 1.13). Addition of a reducing agent, ascorbic acid prevents the increase in MetHb. Addition of glucose and NADH allows the Embden Meyerhof enzyme system in the nano artificial RBC to decrease MetHb further (Fig. 1.13).

Our studies show that using a polyethylene-glycol-polylactide copolymer membrane we are able to strengthen the membrane and increase the circulation time of these nano artificial RBCs to double that of PolyHb (Fig. 1.14) (Chang et al. 2003). The result of other groups support these findings (Zhang et al. 2008; Sheng et al. 2009). We also reported that infusion of 1/3 blood volume into rats did not have any adverse effects on the kidney (Liu and Chang 2008a) or the liver (Liu and Chang 2008b) on a long term basis.

Further studies show that one infusion with a volume of 1/3 the total blood volume, did not result in adverse effects on the biochemistry and histology of the kidney (Liu and Chang 2008a) or liver and spleen (Liu and Chang 2008b). Our most recent study uses PEG-PLA membrane nano artificial cells containing PolyHb-catalase-superoxide dismutase-carbonic anhydrase in a hemorrhagic shock rat model with 2/3 of the blood removed. After one hour of hemorrhagic shock at 30 mmHg, infusion of this preparation effectively resuscitated the animal and lowered the elevated tissue PCO2 (Wei et al. 2013).

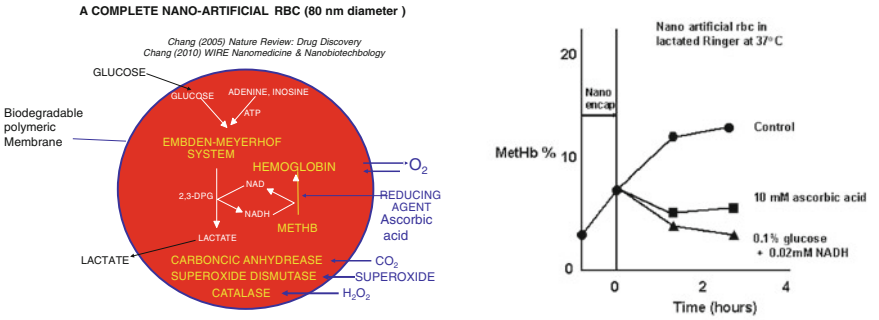


**Fig. 1.13** *Left* Biodegradable polymeric membrane nano artificial RBC contains Hb and all the enzymes of RBC. The membrane is not permeable to larger molecules, but freely permeable to glucose and reducing agents from plasma. *Right* When incubated at 37 °C MetHb increases quickly. Addition of a reducing agent, ascorbic acid prevents the increase in MetHb. Addition of glucose and NADH allows the Embden Meyerhof enzyme system in the nano artificial RBC to decrease MetHb further (with copyright permission from Chang 2007 Monograph on Artificial Cells)

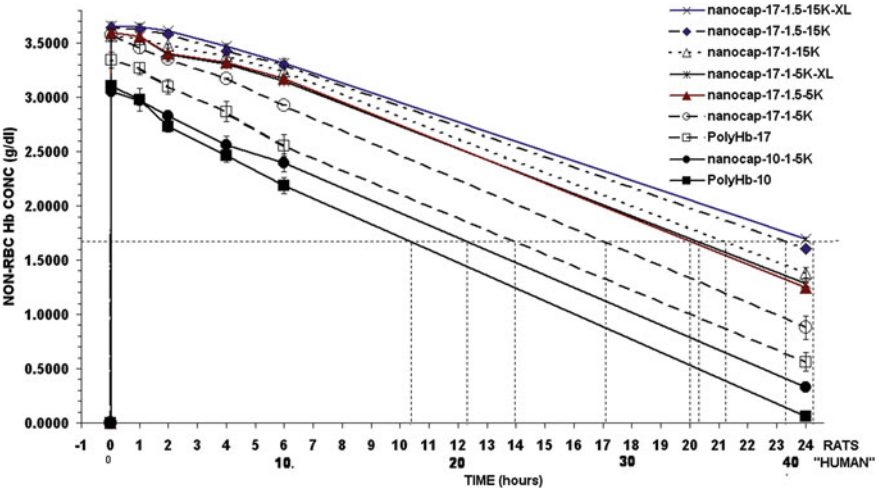


**Fig. 1.14** Comparison of the maximal systemic non-RBC Hb reached after infusion of different preparations and the time to reached a given non-RBC Hb level. The time for PolyHb-17 to reach a non-RBC Hb level of 1.67 gm/dl is 14 h in rats equivalent to 24 h in human. The time for different types of nano artificial RBCs to reach this non-RBC Hb concentration is used to calculate the equivalent time for human (Chang et al. 2003) (with copyright permission from J Artificial Cells, Blood Substitutes and Biotechnology)

### *1.4.4 Possible Variations in the Membrane of Nano Artificial RBC*

Hb lipid vesicles are bilayer membrane nano artificial RBCs containing Hb (Adding PEG to the bilayer lipid membrane greatly increased the circulation time. These PEG-lipid vesicles are more like the lipid–polymer membrane artificial cells (Chang 1972) and are no longer pure lipid vesicles. Discher's group (Photos et al. 2003) tried to increase the strength of the PEG–lipid membrane artificial cells by using self-assembling of block copolymers. PEG was the hydrophilic block and polyethylene or polybutadiene (PB) was the hydrophobic block. This significantly increased strength when compared to PEG–lipid membrane artificial cells. This so-call polymersomes are PEG-PB nano artificial RBC similar to PEG-PLA nano artificial RBC. Thus, polymeric membrane artificial cells have branched off into multilamellar liposome that then has evolved into lipid membrane artificial cells, then polymer-lipid membrane artificial cells, and finally back to the polymeric membrane artificial cells that are now called by different names including polymersomes, nanocapsules, nanoparticles, vesicles and others.

## 1.5 Stem Cells for Blood Substitutes

Some feel that we should depend on stem cells to prepare RBCs. This may be useful for platelets and leucocytes since only small amounts are needed. Even then, platelets, unlike nanobiotechnological derived ones, has extremely short storage life. In the case of RBCs, despite much research, it is still not possible to scale this up sufficient for the large volume of RBC needed (Mazurier et al. 2011). When scale up becomes a reality, this will be an important source of RBC for many clinical conditions. However, these RBC will still have many of the same problems of RBC. These include:

- RBCs need refrigeration but still have a short storage time at 4 °C of less than 42 days. PolyHb can be stored in room temperature for more than 1 year. Freeze-dry powder of PolyHb and PolyHb-enzyme has even longer stability.
- RBCs cannot be freeze-dried into powder form. HBOCs in the freeze dry form are light and compact with ease of transport and storage for emergency, major disaster or war.
- Unlike RBCs, HBOCs can better perfuse obstructed microcirculation as in stroke, heart attack, ischemic limbs, sickle cell anemia and other conditions. It can also better perfuse disturbed microcirculations as in tumour, hemorrhagic shock and other conditions.
- Unlike RBCs, HBOCs can be enhanced with higher enzyme levels than RBCs to be more effective against severe ischemia–reperfusion injury, fatal elevation of tissue pCO2 and other conditions.

- Nanobiotechnology can combine Hb with other enzymes and other bioreactants for specially designed oxygen therapeutics.

## 1.6 Future Perspectives and Learning from Past Experience

The first research on artificial cells was on artificial RBC (Chang 1957, 1964). Yet, instead of blood substitutes, this research has led to extensive extensions and development and medical uses and nonmedical uses in other areas. Examples include nanomedicine, nanobiotechnology, nanotechnology, gene therapy, enzyme therapy, cancer therapy, cell/stem cell therapy, regenerative medicine, liver support support systems, drug carriers, and even in agriculture, aquatic culture, fermentation industry, food industry, nanorobotics, nanocomputers, energy production and other areas (Chang 2007 and www.artcell.mcgill.ca). This is unfortunate, since we are neglecting one of the most important area, blood substitutes and oxygen therapeutics. What are the reasons for this neglect?

There was no blood substitute to replace HIV contaminated blood in the 1980 crisis. Large number of patients receiving donor blood were infected with HIV and died. During this time there was a short period of increase support for basic research and development around the world. Unfortunately we human have short memory and many have forgotten the major disaster that we have encountered and major support for blood substitutes has disappeared after a few years. It is unbelievable that many still think that blood substitute is a simple matter that can be left alone until there is an urgent need. Others think that stem cell derived blood can solve all the problems, but as discussed above, this is not the case. Whatever the excuse, there is little or no interest in supporting blood substitutes research and development at present. Are we waiting again for another crisis to come before repeating our error of doing catch up research and development? The industries have done their best. They have to find their own resources to carry the impossible task of doing everything in basic research, applied research, preclinical studies and clinical trials. With the lack of sufficient resources and with the lack of basic knowledge in this area they have been forced to do trial and error type of R&D with resulting delays and some failures and loss of revenue. Many companies have since given up this impossible endeavor. Without sufficient resources and support for academic basic research and industrial development around the world, development of blood substitutes will continue to be done by trial and error. Furthermore, only the simplest and shortest route will be chosen despite the many possible new generations that are available.

Enormous amount of resources have been placed into basic research and developments on cancer, rare genetic diseases, molecular biology, organ failure and other areas with far less clinically useful results. With far less support, dedicated researchers and developers have persisted beyond any expectation to come

out with clinically useful first generation blood substitutes one of which, PolyHb, has been approved for routine clinical uses in South Africa and Russia. There are a number of others and possible new generation HBOCs. As discussed earlier, first generation Hb oxygen carrier appears to be safe and effective for many clinical conditions. There is no reason to use more complex or more expensive systems in these cases. However, it is not a "cure all" for all clinical conditions. Much research needs to be carried out to study the types of patients who are suitable for this. For instance, we now know that they are not for patients with endothelial dysfunction. They are not for patients with "sustained" ischemia especially if associated with extensive elevation in tissue pCO2. There is nothing in medicine that can be considered as "cure all" and new generations HBOCs need to be develop for those conditions that need these. Thus, much remains to be done before first generation and new generations of blood substitutes can be widely used in more clinical conditions (Liu and Xiu 2008; Mozzarelli 2010; Chang 2013).

Is it reasonable to expect that for blood substitutes, we should be able to come out with a perfect blood substitute with little or no resources for academic and industrial research and development? Should we wait for another crisis before doing catch-up R & D that we now know will not work?

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# Chapter 2
# From the Atmosphere to the Mitochondrion: The Oxygen Cascade

**George P. Biro**

## 2.1 Introduction

Oxygen is essential for energy metabolism whereby the energy trapped in chemical bonds in food stuffs is transferred in a series of stepwise chemical reactions to be available for all cellular functions in the form of high-energy phosphate compounds (adenosine triphosphate and creatine phosphate). Whereas there are energy-generating processes that do not require oxygen, their contribution to the total energy homeostasis is minimal and is of limited applicability.

Food stuffs in the form of carbohydrates and fats are utilized by glycolysis, fatty acid oxidation and the citric acid (Krebs) cycle to generate initially the high energy phosphate compounds NADH and FADH in mitochondria. These are energy-rich because they contain a pair of electrons with high transfer potential. When these electrons are transferred to oxygen, energy is liberated. This energy is trapped with high efficiency in the form of ATP (adenosine triphosphate) by the process of oxidative phosphorylation.

ATP is used in an enormous variety of biological processes comprising both maintenance functions at the basal level (e.g. maintenance of cellular ion channels and gradients) and in energy consuming processes involving external work (such as muscular exercise). Because of the diversity of the intensity of the processes involving external work of the organism, a wide range of energy requirements need to be met. Hence, the oxygen transport system needs to deliver oxygen to organs and cells at rates that accommodate the widely varying metabolic requirements.

The requirements of the oxygen transport system from the atmosphere to all organs, tissues, cells and mitochondria include (Pennock and Attinger 1968):

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1. The processes need to be energy-efficient such that work done by cardiac and respiratory muscles is not wasteful and represents a relatively small proportion of the organism's total energy output.
2. The process needs to be sensitive to fluctuating demands of organs and cellular metabolic activity.
3. The process needs to be responsive to varying metabolic demands of different organs and be capable of matching distribution of blood flow regionally, to various organs and cells according to their function and metabolic demands.
4. The process needs to be efficient in allowing oxygen to penetrate from blood to metabolizing cells and to their mitochondria by diffusion (Pennock and Attinger 1968; Leach and Treacher 1992; Leach and Treacher 1998).

Various cells in various organs utilize oxygen at highly variable rates (Wagner, Venkataraman et al. 2011), and total body oxygen consumption varies over a ten-fold range from rest to maximal levels of exercise (Wagner 2011). The oxygen transport system needs to respond accordingly. When the system fails to supply oxygen to meet the prevailing demand, a state of *hypoxia* is said to exist. It can take a variety of forms which will be explored below.

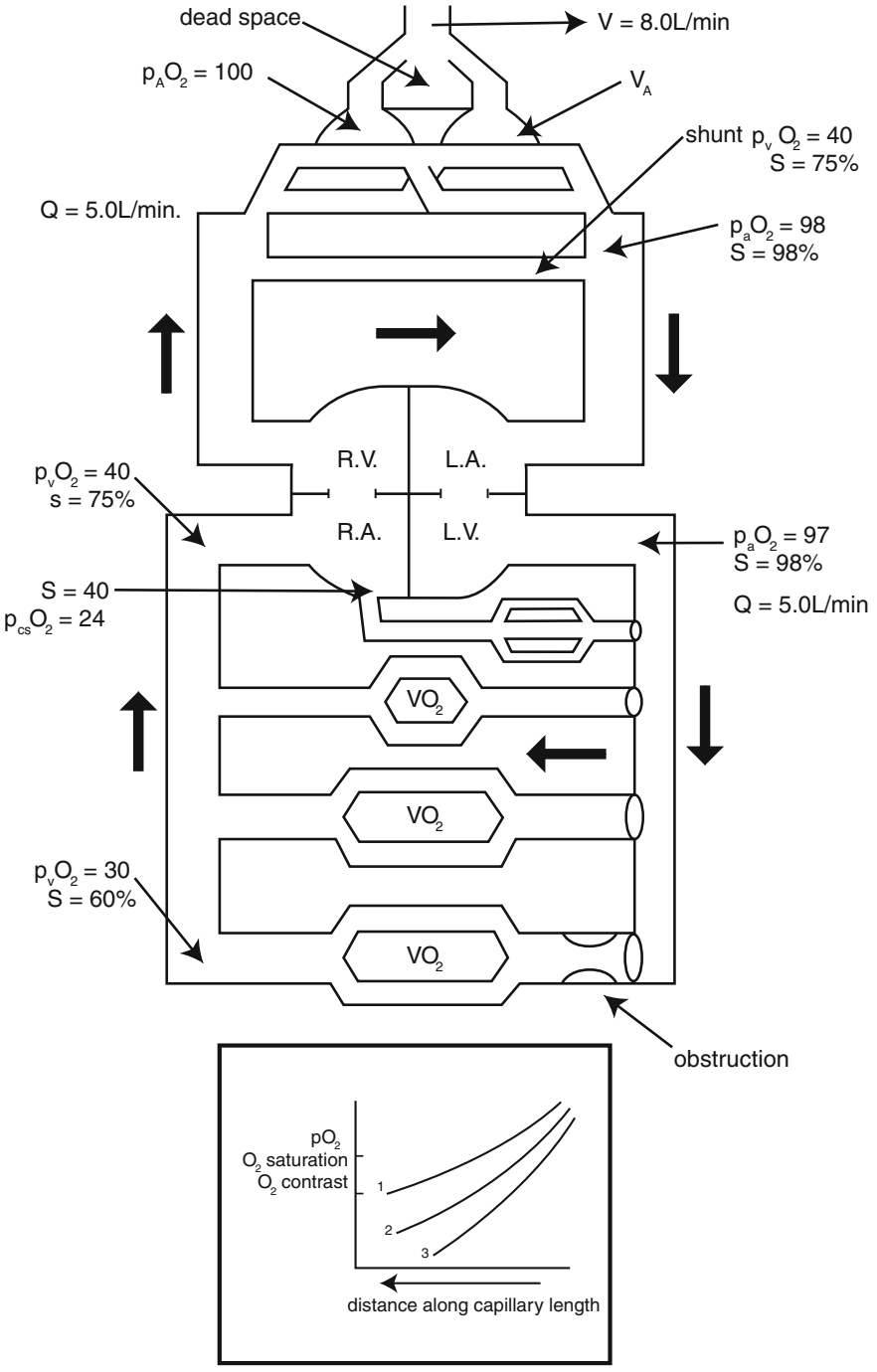
The whole system is schematically illustrated in Fig. 2.1.

## 2.2 The Components of the Oxygen Transport System

The oxygen transport system comprises the following consecutive processes (Leach and Treacher 1992; Treacher and Leach 1998; Schober and Schwarte 2012):

1. Mass transport by active convection of atmospheric air from the environment to the pulmonary alveolar spaces, powered by the contraction/relaxation cycling of the respiratory muscles whose action is regulated mainly by the medullary and pontine respiratory centers and peripheral chemoreceptors.
2. Passive diffusion across the alveolo-capillary membrane, through the plasma and across the erythrocyte membrane and binding to hemoglobin (HGb) "driven" by a partial-pressure gradient for oxygen ($p_AO_2 - pcO_2$).[1]
3. Mass transport by active convection of blood from the alveolar capillaries and the left heart through the vascular distribution system to all systemic capillaries, and return to the right heart, powered by the contraction/relaxation cycling of the myocardium, regulated by the autonomic nervous system, various hormones, vasoactive peptides, prostanoids and nitric oxide (NO) and other local vascular regulatory functions affecting the distribution of blood flow.

[1] The subscripts represent the following: A: alveolar; a: arterial; B: barometric; c: capillary; H2O: water vapor; v: venous.


dead space
V = 8.0L/min
$p_AO_2$ = 100
$V_A$
shunt $p_vO_2$ = 40
S = 75%
Q = 5.0L/min.
$p_aO_2$ = 98
S = 98%
R.V.
L.A.
$p_vO_2$ = 40
s = 75%
R.A.
L.V.
$p_aO_2$ = 97
S = 98%
S = 40
$p_{cs}O_2$ = 24
Q = 5.0L/min
$VO_2$
$VO_2$
$p_vO_2$ = 30
S = 60%
$VO_2$
obstruction
$pO_2$
$O_2$ saturation
$O_2$ contrast
1
2
3
distance along capillary length

◀ **Fig. 2.1** Conceptual schematic of the oxygen transport system comprising the respiratory and circulatory components. The figure includes some of the important, conventionally accepted values of oxygen partial pressures, blood hemoglobin oxygen saturations and gross flow rates of air and blood. In the respiratory system, alveolar ventilation, dead space and shunt components are shown. The schematic illustrates four representative systemic vascular beds, including that of the coronary circulation, showing its high extraction rates of oxygen extraction, and three other representative vascular beds in which oxygen is consumed and extracted from the blood. The bottom vascular bed illustrates one with major obstructions (e.g. atherosclerotic plaque) limiting its flow rates. The insert at the bottom illustrates conceptually the decrements of pO2, oxygen saturation and oxygen content along an idealized capillary under three sets of conditions: 1. normal perfusion; 2. limited perfusion relative to the tissue's oxygen consumption; and 3. inadequate perfusion resulting complete depletion of oxygen before reaching the downstream end of the capillary, resulting in hypoxia of some mass of cells. The abbreviations are: S: oxygen saturation, Q: cardiac output; V: ventilation; VO2: oxygen consumption; subscripts: a: arterial; A: alveolar; v; venous; cs: coronary sinus. RA: right atrium; RV: right ventricle; LA: left atrium; LV: left ventricle.

4. Passive diffusion from the capillary blood across the plasma membrane to the interstitial space, across the cell membrane, throughout the cytoplasm, facilitated by myoglobin (MGb) where present, and into the mitochondria driven by a partial pressure gradient for oxygen (mean capillary $pO_2$ – mean mitochondrial $pO_2$).

There is a critical link between the convective and diffusive phases in the respiratory processes and between the convective and diffusive phases in the circulatory processes. This link is the *oxy-hemoglobin equilibrium relationship,* represented by the oxygen dissociation curve of hemoglobin (ODC), illustrated in Fig 2.2.

*The physiological importance of the oxygen dissociation curve*

Oxygen is dissolved in water governed by a simple linear relationship between oxygen concentration and the partial pressure of oxygen to which the water is exposed (Henry's law). The solubility coefficient of oxygen in water at 37 °C is 0.03 ml/dL/mm Hg. Thus at normal sea-level atmospheric $pO_2$ is about 150 mm Hg, this would result in oxygen concentration in water at body temperature of about 4.4 mL/L (or in more conventional terms 0.44 mL/dL, or 0.44 volume %). This would not permit survival of a human organism. The evolution of hemoglobin with its ability to reversibly bind oxygen to its $Fe^{++}$ iron moiety represents an enormous evolutionary adaptation to homeothermic existence and the ability to meet a wide range of rates of oxygen delivery to meet demands. A more comprehensive exploration of the biochemistry of hemoglobin is presented in a later chapter of this book by Mozzarelli.

Hemoglobin combines proteins (two alpha chains and two beta globin chains) and four porphyrin rings each containing an iron atom within a hydrophobic "pocket" and this arrangement greatly modifies the properties of the iron. When hemoglobin is exposed to very high $pO_2$ all the oxygen binding sites become oxygenated, i.e. binding oxygen molecules. When this occurs the hemoglobin becomes "*fully saturated"* and each *gram* of hemoglobin binds 1.34 ml of oxygen; this is said to be the *"oxygen capacity"* of hemoglobin. At the normal blood

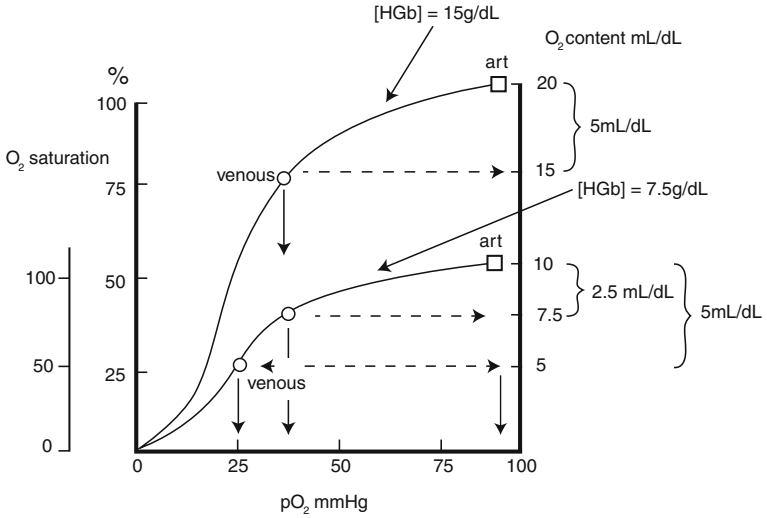



**Fig. 2.2** A conceptual illustration of the oxygen dissociation curves of normal ([HGb] = 15 g/dL) and anemic ([HGb]=7.5 g/dL) blood. Notice that under "normal" conditions at a given flow rate 5 mL of oxygen can be extracted from 100mL of blood resulting in 25% desaturation at a venous pO2 of about 40 mmHg (the presumptive "normal"). Notice also that in the case of anemic blood the extraction of only 2.5 mL oxygen from 100 mL of blood will result in the similar venous blood parameters as in the "normal" case. In order to extract 5 mL of oxygen from 100 mL of perfusing blood requires a lower venous end tissue pO2 and saturation; i.e. greater desaturation. This would only be compensated for by increased blood flow. This is the fundamental basis of the problem in anemia potentially resulting in tissue hypoxia.

hemoglobin concentration of 150 g/L (or more conventionally expressed as 15 g/dL) blood fully saturated with oxygen has an oxygen concentration or *content* of approximately 20 mL/dL. When the blood hemoglobin concentration changes, its oxygen *content* also changes in proportion, but the oxygen capacity of each gram of HGb remains the same. Exposure of HGb to lower $pO_2$ environments results in the "detachment" of some of the oxygen bound, and this is governed by the *Oxygen Dissociation Curve (ODC).*

Oxygen becomes bound to the iron in a sequential manner such that the binding to the first iron atom promotes oxygen binding to the second, and so on, and that the binding to the fourth iron atom requires a greater increment of $pO_2$. The process of unbinding is the exact reverse of this process. These phenomena underlie the "sigmoid shape" of the oxy-hemoglobin dissociation curve (ODC). When oxygen saturation is plotted against the $pO_2$ the curve is initially rising steeply but flattens out as the $pO_2$ rises towards about 70 mm Hg or above, as shown in Fig. 2.1.

Two important advantages follow from this, namely that

1. When blood passes through the pulmonary capillaries it is exposed to the normally high $pO_2$ prevailing in the alveoli (normally about 100 mm Hg at sea level). This is sufficient to nearly fully saturate the hemoglobin. The fact that

the ODC is quite flat in the alveolar $pO_2$ ranges means that even at lower than normal alveolar $pO_2$'s hemoglobin is only slightly desaturated. More importantly, the nature of the oxy-hemoglobin equilibrium relationship greatly facilitates the diffusion of oxygen across the alveolar-capillary wall, because as oxygen diffuses into the plasma, it immediately diffuses into the red cell where it becomes avidly bound to HGb. As a result, the plasma $pO_2$ remains low and there remains a large residual alveolar air-to-plasma $pO_2$ gradient while the intra-erythrocytic $pO_2$ remains low, despite the fact that a substantial amount of oxygen has been added to the HGb component. All of this favors the continuing diffusion of oxygen from alveolar air to capillary plasma and to the red cell HGb. Thus, far more oxygen can be loaded onto and transported by hemoglobin than in simple aqueous solution in blood.

2. When blood passes through systemic capillaries which are surrounded by actively metabolizing cells, this creates a low $pO_2$ environment. At these ranges of $pO_2$ the ODC is quite steep and any small decrease in $pO_2$ results in large decrement in oxygen saturation and release of oxygen first into the plasma and then out to the tissue cells. Here the amplification of diffusion described above is reversed. As oxygen leaves the plasma, $pO_2$ falls and that causes intra-erythrocytic $pO_2$ to fall, as well; the steep part of the ODC ensures that a relatively large volume of oxygen becomes detached from hemoglobin while still keeping the intra-erythrocytic $pO_2$ relatively high. The oxygen exiting the plasma phase is replaced by oxygen desaturation of the HGb within the red cell, thereby maintaining the plasma phase $pO_2$ still high required for continued outward diffusion of oxygen.

The "standard" oxyhemoglobin equilibrium relation (defined at T = 37 °C, pH = 7.4 and $pCO_2$ = 40 mm Hg) is defined as the oxygen saturation of hemoglobin at 50 %, known as the p50, or the oxygen saturation at the midpoint of the saturation scale, or the $pO_2$ required to *half-saturate* HGb. The normal value is approximately 27 mm Hg. The mathematical approximation of the ODC is the Hill equation (see Eq. 2.6 below). While the shape of the curve remains unchanged, a number of factors can modify the *position* of the curve by moving it horizontally either to the right or the left along the $pO_2$ axis. This comes about because of a quantitative change in the *affinity* of hemoglobin to oxygen. The important physiological modifiers of the ODC are temperature, pH, $CO_2$ concentration ($pCO_2$) and the intra-erythrocytic concentration of organic phosphates, particularly 2,3-diphospho-glycerate (2,3-DPG). Increases in each of temperature, $pCO_2$ and a decrease in pH result in a rightward shift, i.e. an increase in the $pO_2$ required to half-saturate HGb, or an increase in p50, also referred to as decreased affinity. A convenient mnemonic is that events occurring in exercising muscle result in a rightward shift, namely *h*ot, *h*acid, *h*ypercapnic. The rightward shift results in *increased unloading* at any given tissue $pO_2$ thereby rendering oxygen unloading more efficient. It also results in a smaller disadvantage in pulmonary oxygen loading, but this is usually obviated by improved ventilation and higher alveolar oxygen tension.

There is insufficient space for a full exploration of physiological importance of the intra-erythrocytic allosteric regulator, 2,3-DPG.

The human red blood cell is uniquely adapted to fulfill a single function, namely gas (oxygen, carbon dioxide, nitric oxide, etc.) transport in that it contains neither a nucleus nor mitochondria. It is "chock full" of tightly packed HGb molecules at a concentration of about 36 g/dL. Hence its energy metabolism depends on glycolysis for the generation of organic phosphates. 2,3-DPG is an intermediate product whose steady state concentration within the red cell is determined by the activity DPG mutase and DPG phosphatase. There is high affinity binding between beta chains of globin and 2,3-DPG. In the presence of a high concentration of 2,3-DPG, a conformational change occurs within the HGb molecule such that the p50 is increased. The range of the change in p50 that can be induced by changes in 2,3-DPG concentration within the red cell is substantial, 15–34 mm Hg (Nunn 1987). Functionally, a change in p50 from 27 to 34 mm Hg means that at a $pO_2$ of 40 mm Hg there is nearly 10 % greater desaturation of HGb giving off about 1.6 ml/dL more oxygen to the tissues (Murray 1976).

The effect of a reduction in intra-erythrocytic pH is partly mediated by a change in 2,3-DPG concentration, by inhibition of DPG mutase and regulation of DPG phosphatase, resulting in a rightward shift of increased p50.

An important distinction must be made between $pO_2$ and oxygen saturation on the one hand and oxygen *content* on the other. As noted above, each gram of HGb when fully saturated (high $pO_2$) is capable of binding 1.36 mL of oxygen. This implies that at the normal HGb concentration of about 15 g/dL blood contains almost 20 mL oxygen/dL. In its passage through an hypothetical vascular bed that takes up 5 mL of oxygen from each 100 mL of blood passing, the venous blood oxygen content will be 15 mL/dL, the venous oxygen saturation will be 75 % (i.e. [20–5]/20 × 100 %), and the venous $pO_2$ will be 40 mm Hg. However if the HGb concentration is reduced to e.g. 7.5 g/dL, the oxygen capacity when saturated will be about 10 mL/dL. In the same hypothetical example above, for example after blood loss and replacement by an infusion of a crystalloid the extraction of 5 ml oxygen/dL of blood passing venous blood oxygen saturation will be 50 %, $pO_2$ will be about 27 mm Hg and tissue oxygen supply will be compromised. Thus, the HGb concentration will define the oxygen capacity and content of the blood, *but not the oxygen saturation* which is defined exclusively by the ODC and the $pO_2$ to which the blood is exposed. This is illustrated schematically in Fig 2.2. In fact, in the example above, the physiological adaptation that ameliorates the potential hypoxia is that the ODC is shifted to right thereby contributing some "extra" oxygen unloaded at any given capillary blood $pO_2$.

What are the physiological consequences in pathological states?

Bank blood stored at 4 °C rapidly loses its 2,3 DPG resulting in a leftward shift and a p50 that may be as low as 15 mm Hg; under these circumstances oxygen unloading in the tissues is quite difficult and it takes at least 24 h for the transfused red cells to "rejuvenate" and to recover normal oxygen affinity. On the other hand, anemia (either chronic or acute) is accompanied by a rise in intraerythrocytic 2,3-DPG, resulting in a rightward shift with improved oxygen unloading in the tissues.

In the presence of severely reduced arterial $pO_2$ (as in high altitude or severe pulmonary disease) the effects are mixed; the rightward shift impairs loading in the lung, but promotes unloading in the tissues. In the presence of respiratory failure and $CO_2$ retention the acidosis and hypercapnia causes a rightward, shift, but when corrected to standard conditions the p50 and 2,3-DPG concentration revert to normal. A number of, but by no means all, inherited hemoglobinopathies manifest themselves by a conformational change in the globin chains and either a rise or fall in p50. Sickle cell anemia is a special case because in addition to a change in its oxygen affinity it combines dramatic changes in red cell membrane deformability and fragility, crystallization of the hemoglobin and a specific vasculopathy.

Three gases form special ligands to hemoglobin. Carbon monoxide is naturally produced in the body during porphyrin catabolism, but exposure to unphysiologically high partial pressures is characterized by displacement of the oxygen from heme iron, because of CO's excessively high affinity to heme (about 300 times that of oxygen). The binding of CO to the heme iron reduces the oxygen capacity and simultaneously causes a leftward shift of the residual oxygen binding. Cyanide similarly has a very high affinity binding to heme iron, and acts similarly to CO, but because of its even greater affinity to cytochromes in the respiratory electron transport chain, it inhibits oxygen consumption and energy production.

Lastly, the normal ligand, nitric oxide, has a very special role in vasoregulation in part through a cycling process between HGb iron and S-nitrosyl groups of globin (Lima, Forrester et al. 2010; Haldar and Stamler 2013). This is an extremely important process in cardiovascular signalling to regulate oxygen delivery.

The following types of hypoxia are recognized:

- Hypoxemia when the oxygen saturation of blood is subnormal;
- Tissue hypoxia when oxygen supply at the organ, tissue or cellular level is inadequate;
- Stagnant hypoxia when tissue hypoxia is caused by inadequate delivery by reduced blood flow (e.g. ischemia);
- Histotoxic or cytotoxic hypoxia when ATP production is impaired or stopped by an agent that interferes with the mitochondrial respiratory chain by competing with and binding to oxygen consuming sites (e.g. cyanide);
- Anemic hypoxia when oxygen delivery is deficient because of inadequate functional hemoglobin concentration of the blood, or reduction of the oxygen capacity by "occupation of" a significant proportion of binding sites by other ligands with higher affinity than that of oxygen.

The physiological consequences of hypoxia are far too numerous even to enumerate here. Suffice to say that they depend on its severity, the organism's tolerance and whether the hypoxia is sudden and short-lived, or is long standing such that adaptations and tolerance have developed to ameliorate some of its effects. Cellular tolerance of hypoxia may involve a variety of mechanisms, including "hibernation" to reduce metabolic activity, increased extraction of oxygen and adaptations of enzyme systems and gene expression to permit metabolic activity at lower levels of oxygen availability(Leach and Treacher 1992).

The following equations represent the components and their interactions of the system (Leach and Treacher 1992).

$$\textbf{Alveolar air } pO_2: \quad p_AO_2 = F_iO_2 \times (P_B - P_{H_2O}) - (p_ACO_2/RQ) \tag{2.1}$$

$$\textbf{Blood oxygen concentration}: \quad C_aO_2 = S_aO_2 \times [HGb] \times 1.36\} + p_aO_2 \times 0.0031 \tag{2.2}$$

$$\textbf{Oxygen delivery rate}: \quad DO_2 = QX\,C_aO_2 \tag{2.3}$$

$$\textbf{Oxygen extraction ratio}: \quad EO_2 = VO_2/DO_2 \tag{2.4}$$

These equations represent the following:

(Equation 2.1) *The alveolar air equation* represents the partial pressure of oxygen in alveolar air at the prevailing barometric pressure after accounting for the vapor pressure of water with which tracheal air becomes saturated at body temperature. It defines the oxygen partial pressure in the steady state accounting for oxygen extracted and $CO_2$ added by the respiratory gas exchange. This is the oxygen partial pressure with which blood in the pulmonary capillaries equilibrates during its rapid transit through the capillary. This oxygen partial pressure defines the oxygen saturation of hemoglobin according to the oxygen dissociation curve (ODC).

(Equation 2.2) Describes *blood oxygen concentration or content in the arterial blood* that arrives from the pulmonary circulation (not accounting for any loss through venous admixture). The oxy-hemoglobin saturation, as defined above, is the arithmetic product of the oxygen capacity of 1 gram of hemoglobin when fully saturated (1.36 mL/g) and the hemoglobin concentration, plus the additional amount of oxygen dissolved in physical solution in blood water at body temperature (0.0031 $mLO_2$/mL of water/mm Hg). It clearly shows the superiority of hemoglobin binding of oxygen over oxygen dissolved in the aqueous compartment of blood. The presence of 1g of hemoglobin when fully saturated multiplies oxygen transported by blood by over 40-fold. Fifteen g hemoglobin/100 mL of blood multiplies oxygen transported over 600-fold. The two equations combined illustrate the advantages of breathing oxygen enriched air mixtures when hemoglobin is not fully saturated when breathing atmospheric air, or at high altitude.

(Equation 2.3) Defines *the oxygen delivery rate* that describes the equal importance of the cardiac output and of the oxygen content of the blood in satisfying the oxygen need of the organism in a complementary manner. It does not, however, address the fine tuning of delivery required to satisfy varying oxygen demands of the various organs and how these may be satisfied by modulating the *distribution* of the cardiac output. When this equation is applied to an individual organ, the CO is replaced by the organ blood flow and this is the variable that is modulated over a wide range to meet the needs of the organ in question.

Implicit in the *Oxygen Extraction equation* (Eq. 2.4) is the fact that the proportion of oxygen that can be extracted from blood is finite and a significant concentration of oxygen must remain in blood returning to the venous system. The extraction ratio defines the residual oxyhemoglobin saturation, and according to the ODC, the oxygen partial pressure after the blood's transit through the systemic capillary beds. A finite $pO_2$ must exist at the end of the capillary bed to assure that a sufficient $pO_2$ gradient exists to maintain diffusion of oxygen from the capillary to cells at some distance.

At rest, the blood returning from the systemic circulation is normally about 70–75 % saturated and has a mixed venous $pO_2$ of approximately 40 mm Hg. Some organs extract a good deal more of the oxygen (e.g. the heart), while others extract less; some vary their extraction over a very wide range (e.g. skeletal muscle at rest and during exercise), whereas others (e.g. brain) maintain global oxygen consumption and extraction nearly constant.

The following sections will describe the four stages of the "oxygen transport system" and the 'oxygen cascade" from the atmosphere to the mitochondria.

It is very important to note that the critical link between the convective mass transport and diffusive processes in both the respiratory and circulatory phases of oxygen transport is the *oxy-hemoglobin dissociation (ODC)* relationship between the partial pressure of oxygen ($pO_2$) and the saturation of the hemoglobin and thereby the oxygen content of the blood. The Chapter by Mozarelli describes the mechanisms underlying and the relationship itself in great detail. For the current purposes it is important to note that the ODC greatly facilitates the loading of oxygen onto HGb in the pulmonary circulation exposed to high alveolar $pO_2$, and the unloading of oxygen in the capillaries in the systemic circulation by the continuous exposure of the blood to lower extravascular $pO_2$ by the continuously consuming cells in the capillaries' immediate environment.

## 2.3 The First Step in the Oxygen Cascade

### *2.3.1 Mass Transport from Environment to Alveolar Space*

Atmospheric air containing 21 % oxygen at a total atmospheric pressure of 760 mm Hg at sea level has a $pO_2$ of approximately 159 mm Hg. Within the tracheo-bronchial tree the air is warmed to body temperature and saturated with water vapor which reduces the effective air pressure by 47 mm Hg; thus air entering the alveolus has a $pO_2$ of (713 mm Hg × 21/100) about 150 mm Hg. $CO_2$ is added to alveolar air at a partial pressure of 40 mm Hg, thereby diluting the alveolar air oxygen concentration and reducing the mean alveolar air $p_AO_2$ to approximately 100 mm Hg, as described above by the alveolar air equation (Eq. 2.1.).

#### 2.3.1.1 Pulmonary Ventilation

Respiratory muscle activity of inspiration/expiration cycling maintains two–way airflow and averaged over several cycles, maintains a partial pressure of oxygen and carbon dioxide in the alveolar air of 100 and 40 mm Hg, respectively. In the face of marked changes in the metabolic consumption of oxygen and production of carbon dioxide the alveolar $pO_2$ and $pCO_2$ are maintained remarkably constant at these values by complex neural *regulation of the total and alveolar ventilation.*

Metabolic oxygen demand and carbon dioxide production vary from basal needs during restful sleep to maximal levels of exercise, over an approximately tenfold range (Murray 1976). A centrally located "oscillator" generates reciprocal stimulation/inhibition of inspiration/expiration cycling, located in the pons (Morschel and Dutschman 2009). This central rhythm is modulated in the medulla oblongata where the principal mechanisms regulating both respiration and the cardiovascular system are located. The principal respiratory regulation whereby arterial blood $pCO_2$ is maintained resides in an area on the medulla's ventral surface which is exquisitely sensitive to changes in pH, primarily resulting from unbuffered changes in $pCO_2$ in the cerebro-spinal fluid (CSF) bathing this region (Guyenet 2008; da Silve, Li et al. 2010; Nattie 2011). Minor changes in $CO_2$ in this region bring about marked changes in alveolar ventilation that result in maintenance of a near-constant alveolar and arterial blood $pCO_2$. The neural output from the medullary respiratory centers is conveyed by efferents from spinal motor nuclei to the diaphragm, intercostal and abdominal muscles. In addition to the medullary respiratory centers, neural input is conveyed by afferent fibers in the vagus (X) and glosso-pharyngeal (IX) nerves from the aortic and carotid body chemoreceptors to the nucleus of the solitary tract and then to the pontine and medullary integrative neurones. The aortic and carotid chemoreceptors are principally sensitive to significant changes in the $pO_2$ of arterial blood, but are also sensitive to changes in $pCO_2$. These chemoreceptors are primarily responsible for driving ventilation in response to reduction in the arterial blood $pO_2$, that occurs e.g. at high altitude or acute pulmonary injury. In addition, feedback information on lung deformation by stretch receptors is conveyed from parenchymal mechanoreceptors by the Vagus nerve, and from respiratory muscles from muscle spindles via afferents to the spinal cord.

In order to generate airflow into the lung, inspiratory muscle contraction overcomes three forces: 1. The elastic recoil of the lung and chest wall complex; 2. The frictional resistance to airflow in the airways and the frictional resistances between lung and thorax; and 3. Inertial airflow resistance which is primarily determined by the aggregate cross sectional area of the airways, according to Poisseuille's law (Murray 1976). The normal airways at resting breathing offer minimal resistance and the work of breathing represents a small proportion of the total body oxygen consumption. However, when an increase in ventilation and oxygen delivery are required by increased metabolic activity, the work of breathing is also increased. As ventilation increases by both augmented tidal volumes and respiratory rates, bulk airflow and flow velocity within the lung in

both directions is increased exponentially, the flow becomes turbulent, and the work of breathing becomes one of the limiting factors at maximal exercise because it consumes all the additional oxygen intake. Likewise, at near-maximal levels of ventilation expiratory muscle effort is required to maintain the high-velocity airflows at fast respiratory rates. The expiratory muscle contraction under these conditions may lead to dynamic compression of airways, especially those not fully supported by cartilage. The resulting compression increases airway resistance further, leading to deleterious positive feedback and air trapping. The resistance offered to airflow is varied according to the degree of contraction or relaxation of airway smooth muscle, sensitive to sympathetic innervation and agonists, various inflammatory mediators and NO-mediated dilators (Spina 2008). However under pathological conditions characterized by excessive airway smooth muscle contraction (e.g. asthma) airway resistance may increase many-fold increasing the work of breathing, even at low levels of ventilation, to an extent that it becomes a very large proportion of total body oxygen consumption (Ozier, Allard et al. 2011). Thus, pathological airway responses may impose major limitations on the ability of the oxygen transport system to respond to increased demands and thereby limiting exercise capacity (Ozier, Allard et al. 2011). The airways themselves are also capable of sensing oxygen and respond appropriately in a reflex manner (Peers and Kemp 2001; Waypa and Schumacker 2010).

The phenomenon of hypoxic pulmonary vasoconstriction is observed when alveolar air hypoxia occurs; this is observed globally at high altitude and locally when some lung units are hypoxic because of reduced local alveolar ventilation. This assures that blood flow is diverted to areas of the lung that are better ventilated. The mechanism is not fully known, but it involves oxygen sensing by vascular (probably endothelial) cells, signalling to trigger a functional response (Waypa and Schumacker 2010).

#### 2.3.1.2 Pulmonary Blood Flow

The whole of the cardiac output (with the exception of the bronchial (arterial) flow) is driven by right ventricular contraction through the entire pulmonary circulation. At the inlet, pulmonary arterial pressure at rest is normally approximately 20/10 mm Hg and at the outlet, left atrial pressure is about 5−8 mm Hg and the mean pressure loss across the pulmonary circulation is about 12 mm Hg. The mean capillary pressures are estimated to be of the order 10−12 mm Hg, significantly lower than those in systemic capillaries. This assures that: 1. The alveolar spaces are kept dry by Starling forces. 2. The structural integrity of the alveolocapillary membrane is not disrupted. 3. The total resistance offered by the pulmonary circulation to blood flow is about one-fourteenth that of the high-pressure systemic circulation, permitting a low level of work by the right ventricle. As estimated from measurements of lung diffusing capacity, at any given time the entire active pulmonary capillary bed contains about 70 mL of blood (Ceridon,

Beck et al. 2010) distributed over an enormous surface area where blood and air are in intimate contact.

#### 2.3.1.3 Ventilation: Perfusion Matching

Two components of the system, normally of minimal magnitude, do not participate in respiratory gas exchange: the *dead space* and the *shunt* components (see Fig. 2.1). The former comprises the anatomical dead space of the large airways and unventilated alveoli. The latter comprises true anatomical right-to-left shunts, and un-perfused alveoli, cumulatively referred to as *venous admixture*. In these components there is no exposure of blood to alveolar air. As long as these components are of negligible magnitude, respiratory gas exchange proceeds efficiently. Normal, fully efficient oxygenation of the blood requires that ventilation and blood flow be evenly matched throughout the lung and in nearly all lung units (West 1965). If perfusion is not matched to ventilation in individual lung units a mismatching occurs with deleterious effect upon gas exchange. When a lung unit is underventilated relative to its perfusion, the alveolar air and end-capillary $pO_2$ will be less than normal. When a lung unit is under-perfused relative to its ventilation, the alveolar air and end-capillary blood $pO_2$ will be above-normal. Thus collectively, under-ventilated lung units behave as shunts, and the underperfused lung units collectively behave like dead space. When there is significant mismatching of ventilation and perfusion of a substantial number of lung units, hypoxemia will result (West 1965).

It may appear counter-intuitive that over-ventilated lung units with above-normal $pO_2$ do not compensate for the effect of under-ventilated lung units whose $pO_2$ is below-normal. The explanation lies in the oxygen dissociation curve (ODC).

Above-normal $pO_2$ will not increase blood oxygen saturation and content because this already occupies the flat part of the ODC and full saturation is achieved at normal $pO_2$. Subnormal $pO_2$ on the other hand will result in reduced saturation and oxygen content. When blood is collected in the pulmonary veins from all lung units including those with mismatched ventilation and perfusion, the mixture will reflect the effect on the ODC of the subnormal blood oxygen content and saturation. The result is reduced oxygen saturation, content and $pO_2$ in arterial blood. While some local mechanisms are operative to regulate ventilation and perfusion of lung units such that ventilation and blood perfusion are more evenly matched (West 1965), for example by hypoxic pulmonary vasoconstriction locally, very significant mismatching of ventilation and perfusion ($V_A/Q$) by lung pathology results in hypoxemia and a corresponding effect on the oxygen transport system (West 1965), as indicated by Eq. 2.3.

## 2.4 The Second Step in the Oxygen Cascade

### *2.4.1 Diffusion from Alveolar Air to Blood in the Pulmonary Capillary*

Four factors are important in efficient respiratory exchange at rest between alveolar air and capillary blood in the lung: 1. A large $pO_2$ gradient of approximately 100−40 mm Hg; 2. A large surface area available for gas exchange with a thin diffusion barrier; and 3. A favorable diffusion coefficient for oxygen. These three factors facilitate the near-complete equilibration of oxygen partial pressures during the rapid (approximately 0.7 s.) transit time of blood through the capillary bed. The fourth factor, the ODC facilitates the transfer of a large amount of oxygen by diffusion from alveolar air to solution in plasma and into the red cells by *rapid binding* to HGb. The initial rapid diffusion of oxygen is promoted by the high oxy-HGb affinity on the steep part of the ODC when the partial pressure gradient is greatest. As the blood arrives in the pulmonary capillary with a mixed venous $pO_2$ of about 40 mm Hg and about 70−75 % saturated, the binding of the fourth oxygen atom is fastest. As the gradient declines along the passage of blood through the capillary's length, equilibration occurs less rapidly, but is promoted by the flat top part of the ODC, even at varying alveolar oxygen partial pressures (Murray 1976; Nunn 1987). Equilibration of $pCO_2$ between alveolar air and capillary blood is even more rapid than that of oxygen, even at smaller partial pressure gradients (46−40 mm Hg), because of the higher diffusion coefficient.

When metabolic demands increase, as in exercise, fever, etc. both cardiac output and ventilation respond. This results in increased bulk flow and flow velocity of both air in airways and blood in the pulmonary circulation. The limitations thereby imposed on respiratory gas exchange (e.g. shortened capillary transit time) are ameliorated by a number of factors, including:

1. Recruitment of both alveolar surfaces and capillaries, and improved matching of ventilation and perfusion.
2. Widening of the alveolar-to-capillary partial pressure gradient by virtue of the increased oxygen extraction in the systemic circulation and the reduced venous blood $pO_2$ in the blood returning to the lung.
3. Rightward shift in the ODC of venous blood entering because of the lower blood pH and $pCO_2$.

This second step of diffusive gas exchange occurs passively, at the cost of moving air and blood by the work of the respiratory muscles and of the right ventricle satisfying the requirement of efficiency as noted above.

The processes in these first two stages of oxygen transport optimize the content component of Eq. 2.3 above. Important impairments would be represented by

1. Impaired respiratory function (neuro-muscular impairment, chest wall deformities, pleural masses, reduced lung compliance).

2. Reduced barometric pressure.
3. Pulmonary parenchymal or vascular disease.
4. Cardiac disease.
5. Central nervous system and spinal cord injuries or disease (e.g. motor neuron diseases).
6. Deficiency of functional hemoglobin (anemia, metHGbemia, carbon monoxide poisoning, hemoglobinopathies).

**In summary**: Pulmonary ventilation is regulated by altering the neural outputs to changing both the frequency and tidal volume. These changes result in alterations of the alveolar ventilation and thereby the volumes from which oxygen is extracted and carbon dioxide is added changing the steady state concentration of gases with which blood in the pulmonary capillaries equilibrates. Pathological conditions affecting the ability to maintain normal blood gas composition may have a major impact on the ability of the organism to provide adequate oxygen supply to meet all metabolic requirements of organs and tissues, resulting in hypoxia.

## 2.5 The Third Step in the Oxygen Cascade

### *2.5.1 Mass Transport from the Pulmonary to the Systemic Capillaries*

#### 2.5.1.1 The Cardiac Output

The cardiac output (CO) is the volume of blood pumped by each ventricle of the heart expressed as L/min. Its two components are the heart rate (HR) and the stroke volume (SV); the typical range being about 60 times/minute × 75 mL = 4,500 mL/min. From basal level at rest to maximal exercise the cardiac output can vary over an approximately five-fold range to about 22.5 L/min, by increasing HR over an approximately three-fold range and the SV over an approximately two-fold range. Thus, oxygen transport can be varied to meet a wide range of metabolic demands, (see Eq. 2.3.) and by modulating the distribution of flow among organs according to their activity. Thus, at rest blood flow to skeletal muscle is very low, but during vigorous exercise blood flow is augmented disproportionally to the exercising muscles and to the heart, and may be maintained or even temporarily curtailed to some organs, such as the abdominal organs. Alternatively, during digestion blood flow to the gastro-intestinal tract is increased to meet the secretory and absorptive needs. This permits the "economical" and efficient conservation of cardiac work required to augment the cardiac output satisfying the requirement described above.

### 2.5.1.2 The Regulation of the Heart Rate (HR)

The rate of spontaneous depolarization arising in the sino-atrial node in the right atrium determines the heart rate under normal circumstances. It is under the influence of the sympatho-adrenal system accelerating it and its vagal innervation slowing it. Normally, at rest it is under a predominant vagal influence and responds by increasing the HR both by inhibition of the vagal and augmenting the sympathetic influences. One of the clinically observable phenomena is the reciprocal HR response to an acute change in blood pressure mediated by the *baroreceptor reflex or baroreflex;* the receptors being located in the carotid sinus and the aortic arch. As the HR is accelerated under tachycardic conditions, ventricular function is altered *indirectly,* by shortening the time available for both filling and ejection. The ventricles adapt to such changes by altering their *contractility* (see below). The HR is not *directly* affected by the other mediators that are important in modulating ventricular function and, thereby the SV ejected.

### 2.5.1.3 The Regulation of the Stroke Volume (SV)

The determinants of the stroke volume produced can be described by terms borrowed from skeletal muscle mechanics: *preload, contractility and afterload*. Preload in this respect is represented by the end-diastolic volume of the ventricle. The afterload is represented by the load against which the contraction generates force; in this case the aortic and PA pressures, respectively for the left and right ventricles. As long as the rising intraventricular pressure is less than aortic and PA pressures, the contraction is isovolumic and myocyte shortening only stretches elastic connective tissue elements. Once intraventricular pressure reaches aortic and PA pressures, external shortening and ejection can begin. Thus the contraction does internal and external work. The total work is determined by all three elements: preload, afterload and the particular characteristic of the myocardium, its contractility (Hunter, Janicki et al. 1980).

There are two important mechanisms whereby the myocardium is capable of varying its tension generation and the magnitude of the stroke volume ejected: 1. That based on *Starling's Law* of initial length which is an inherent property of the heart itself and is determined principally by the preload; and 2. Changing *contractility* by external influences, namely the ability of changing the work performed from a given preload, against a given afterload (Sagawa, Suga et al. 1977; Baan, van der Velde et al. 1992).

*Starling's law* states that, over a certain range, the force generated by the ventricle and thereby the stroke volume ejected is related to the preload i.e. the volume contained in the relaxed ventricle at the end of filling, the *end-diastolic volume*. The underlying mechanism is related to the "sliding filament theory" of muscle contraction in that the extent of the overlap of the actin and myosin filaments before contraction begins determines the number of cross bridges that

can be formed and, consequently the magnitude of the force generated and work done.

In the intact organism changes in *contractility, or of inotropic state*, are of far greater significance than the Starling mechanisms. A change in *contractility* occurs under the influence of sympatho-adrenal stimulation, inotropic drugs, and is represented by an increase in the forces generated at any given preload and afterload. It is manifested by: 1. An increase in tension generated from the same preload, against a given afterload. 2. A greater *velocity* of shortening. 3. An increased stroke volume ejected from the same end-diastolic volume by contraction to a smaller end-systolic volume. The subcellular mechanism underlying increased inotropic state likely involves a rise in intra-cellular calcium concentration in systole, resulting in the greater engagement and faster cycling of more of the cross bridges participating. Of the two mechanisms, the Starling mechanism is involved when the increasing the inotropic state is not possible, namely in heart failure (Norman, ouriri et al. 2011; Little and Applegate 1993). Otherwise, the preferred mechanism to increase stroke volume is by changes in the *inotropic state* or *contractility*.

At normal heart rates the *duration of diastole* provides sufficient time for adequate filling to occur, but at elevated heart rates the period of diastolic filling is shortened and atrial contraction is required for adequate filling to occur, and sufficiently high diastolic compliance, i.e. ease of extension of the chamber, is of critical importance. During periods of sympatho-adrenal stimulation both heart rate and stroke volume are increased, the latter by the augmented contractility whereby the ventricle is capable of augmenting the stroke volume by both increased velocity of shortening and contraction to a smaller end-systolic volume in the shortened time available for a cardiac cycle. Thus, whereas tachycardia increases the number of times a stroke volume is ejected and, coincidentally limits the duration of each cardiac cycle, the simultaneous increase in contractility permits substantially increasing the stroke volume, thereby achieving a maximal cardiac output range of five-fold over basal.

The continuous flow of blood to the left heart is "boosted" at high pressure by left ventricular contraction and is propelled through the arterial distribution system to all the organs and to their capillaries. The distribution of blood flow to individual organs, and within them regionally, is under a complex regulatory system "designed" to optimize oxygen delivery to meet the organs' metabolic needs and respective non-metabolic functions. The regional distribution of the cardiac output (CO) is regulated by the respective organs' aggregate resistance comprising the degree of contraction/dilation of the smooth muscle of the arterioles. The principal regulators of arteriolar smooth muscle contraction/relaxation are the autonomic nervous system, various vasoactive peptide hormones (including endothelin and angiotensin), and the locally acting "autacoid" prostaglandins (including prostacyclin and thromboxane), as well as the local $O_2$ and $CO_2$ concentrations. The principal mechanism whereby relaxation of arteriolar smooth muscle is mediated involves the generation of nitric oxide (NO) which regulates the function of the intracellular enzyme soluble guanylate cyclase (sGC), the product of which, cyclic

guanosyl monophosphate (cGMP) in turn, inhibits cross-bridge cycling of vascular smooth muscle cells. The complexity of the regulation of blood flow in each organ is expressed at a variety of levels: the phenotypic diversity of vascular smooth muscle cells and their receptors specific to each organ, the type and density of receptors responsive to vasoactive peptides and to autonomic nervous system mediators, the hierarchy of all of these regulatory functions, and finally the respective organ's metabolic demand for oxygen and demand for blood flow specific to the organ's non-metabolic function (e.g. secretion, absorption, regulation of water and ionic composition and temperature).

**In summary**: The cardiac output is regulated through induced changes in the heart rate and the stroke volume. The heart rate is regulated by the sympathetic and parasympathetic systems; sympathetic influences have a positive chronotropic, whereas, through the vagal innervation parasympathetic influences have a negative chronotropic effect. The ability to alter contractility is a critically important property of the heart; in the face of effects that would limit the range of possible stroke volumes (reduced preload by encroachment on filling by a shortening of the diastolic period, reduction of the external shortening possible by increased afterload), it permits extending the range of stroke volume by increased tension generation at greater velocity and contraction to smaller end-systolic volumes.

As shown by Eq. 2.3. the cardiac output which can be varied over a five-fold range, is the central variable component in systemic oxygen transport. Normally, the oxygen content is relatively fixed and, unlike the cardiac output, is not variable on a moment-by-moment basis. Hence, the cardiac output is the principal variable whereby the deficiencies in the oxygen content of the blood can be compensated for to maintain or augment systemic oxygen transport.

## 2.6 The Peripheral Circulation

The regulation and function of the major components, the major arteries, the arterioles, the capillaries and veins are different and are discussed sequentially.

*Blood flow in major distribution vessels, the arteries*

The major distributing arteries have relatively thick walls and spirally arranged smooth muscle under sympathetic control. The function of the smooth muscle is largely to alter the compliance of these vessels and thereby to affect the profile of the pressure wave and the magnitude of the pulse pressure (i.e. the difference between systolic and diastolic blood pressures). The flow of blood is largely streamlined and these vessels offer relatively little resistance to blood flow. Hence, there is little pressure dissipated from the aorta to the smallest distribution arteries. Streamlined flow may become turbulent (with accompanying increase in flow resistance) at bifurcations and sites of partial obstructions protruding into the lumen. Branchings of the distributing arteries feed the various organs which are arranged in parallel, and flow to each organ is determined by the relative aggregate resistance the organs' arterioles present.

*Blood flow and pressure in the arterioles*

The arterioles arise from parent vessels by multiple branching to reach a size of approximately 100 μm in diameter. These vessels are invested by a relatively thick circular smooth muscle which is highly responsive to numerous vasoactive mediators acting through a variety of specific receptors. The degree of contraction of the smooth muscle determines the diameter of the arteriole, and thereby the resistance to flow it offers, according to Poisseuille's law (blood flow is directly proportional to the driving pressure and inversely proportional to the radius$^4$, the length and the viscosity of the fluid). In the present context, the single most important determinant of flow at any given driving pressure is the resistance due to changes in arteriolar radius. Thus, the level of the arterioles is the site of largest drop in perfusion pressure. The high-velocity flow in the arterioles is slowed down substantially in the arterioles.

The resistance in the arterioles serve three separate functions:

1. All the arterioles in the aggregate limit outflow from the major arteries, thereby maintaining high mean and diastolic arterial blood pressures.
2. Arterioles of each organ in the aggregate regulate the distribution of blood flow to individual organs.
3. Each arteriole in any given organ reduces the high arterial blood pressure to a level similar to the colloid oncotic pressure of the blood, thereby limiting the pressures to which the poorly supported capillary walls are exposed and facilitating the transcapillary fluid exchange based on Starling forces.

Arteriolar smooth muscle is under complex bi-directional control. It responds by *active contraction* (by increased cross bridge cycling) to a plethora of systemic autonomic nervous, as well as humoral systemic (endocrine: angiotensin, endothelin and local (paracrine: prostanoids) mediators, through a number of diverse receptor types distributed with great organ-to-organ variability. The opposite effect, dilation, is mediated principally by a single mechanism, namely the *inhibition* of cross bridge cycling. The common pathway of dilation involves the synthesis and release of NO which signals locally and downstream the action of soluble guanylate cyclase (sGC) to generate cyclic adenosine monophosphate (cAMP) which is the mediator effecting the inhibition of cross bridge cycling in the smooth muscle and consequent relaxation. In the critical organs with high metabolic activity (brain, heart, kidney, liver) the arterioles are also subject to additional controls regulating the arteriolar smooth muscle activity, sensitive to oxygen availability within the organ, the oxygen sensor likely being the mitochondria (Waypa and Schumacker 2010).

*Blood flow in capillaries*

Because of the high resistance in the arterioles, blood flow velocity is further reduced in the capillaries to facilitate gas exchange. The capillary walls consist of a single layer of endothelial cells supported by a basement membrane. The internal diameter is similar to, or even smaller than, the red blood cell (RBC) so that the cells need to deform in their transit through the capillary. The deformation serves

to mix the RBC contents thereby facilitating even distribution of oxygen and carbon dioxide within. The deformation required to permit RBC passage is also the cause of marked decline of the hydrostatic pressure along the length of the capillary. According to the Starling hypothesis, as a result of the hydrostatic pressure exceeding the colloid osmotic pressure difference across the capillary surface, there is net filtration along the first half of the capillary's length. As the hydrostatic pressure declines below that of the net colloid osmotic pressure across the capillary wall, reabsorption occurs along the distal half of the capillary, with a net zero balance in trans-capillary fluid exchange and a continuous circulation of extracellular fluid.

## 2.7 The Fourth Step in the Oxygen Cascade

### *2.7.1 Diffusion of Oxygen from Capillary Blood to Metabolizing Cells and Within the Cell to the Site of Consumption, the Mitochondria*

The Fick equation of diffusion of a gas in a liquid medium describes the determinants of the flux as

$$\mathbf{V} = \mathbf{D} \times \mathbf{A} \times \{\Delta \mathbf{P} / \mathbf{d}\} \tag{2.5}$$

where A is the area available for diffusion; D is the diffusion constant for the gas; **ΔP** is the gas partial pressure difference; d is the diffusion distance, thus **ΔP/d** is the partial pressure gradient.

The simplest model of oxygen delivery is the Krogh model of two concentric cylinders; a cylinder of cells penetrated centrally by a capillary, approximately equidistant from all cells at the periphery of the tissue cylinder. This simplified model is useful to illustrate the maintenance of diffusive gas exchange along the same principles already described above for pulmonary capillary gas exchange. Oxygen is unloaded from hemoglobin and carbon dioxide is taken up (by three different forms of carriage) and a continuous diffusive flow of oxygen is maintained to all the cells within the capillary's territory. The functional (i.e. open) capillary density determines the diffusion distances from capillary blood to consuming cells and their mitochondria over a wide range of consumption rates, depending on their level of activity (Wagner, Venkataraman et al. 2011). As described above, the presence of HGb in the red cells facilitates diffusion by virtue of taking up oxygen from the plasma and binding, minimizing the increment of oxygen partial pressure per unit volume of oxygen received and extending the wide partial pressure gradient.

The blood entering the capillary with a high $pO_2$ begins to surrender its oxygen because it is surrounded by an immediate environment of lower $pO_2$, initially giving off oxygen dissolved in plasma, and followed by release of oxygen bound to

HGb. Because of the continuous consumption of oxygen by all the cells within the larger cylinder, the partial pressure of oxygen declines both longitudinally and radially from the "inflow" to the "outflow" end (see bottominset in Fig. 2.1). The principal force driving diffusion is the gradient in $pO_2$ from blood to the cells. The oxygen dissociation characteristics of HGb facilitate the rapid and efficient unloading of oxygen within the capillary. The cells at the extreme end of the cylinder and furthest from the capillary are at the greatest disadvantage, because their immediate environment is at the lowest $pO_2$.

The oxygen sink, the mitochondria, represent the extreme end of the diffusion path and the lowest local $pO_2$. Mitochondrial $pO_2$ in vivo is difficult to determine (Lanza and Sreekumaran Nair 2009, 2010), but it has been estimated in one study, using a novel methodology, to be in the range 30–40 mm Hg in vivo in the liver(Mik, Johannes et al. 2008), far higher than the critical $pO_2$ estimated earlier (Lanza and Sreekumaran Nair 2009). If this is indeed the case, then end-capillary $pO_2$ in the same range would be insufficient to drive diffusion from the distal parts of the capillary where the prevailing $pO_2$ may be as high as 30–40 mm Hg (Pitman 2011).

Of course, the Krogh cylinder model as the single source of oxygen for a collection of contiguous cells is a gross oversimplification of the real world of the complex networks of the microcirculation with continually opening and closing of individual capillaries and at any time there are excess capillaries supplying more than a single collection of cells. The arrangement facilitates altering the diffusion distances from capillary to cells and thereby optimize oxygen diffusion to the cells and mitochondria (Pitman 2011). The gradients existing in the microcirculation are far more complex than those illustrated by the Krogh cylinder model (Tsai, Johnson et al. 2003; Pitman 2011). Nevertheless, *directional changes* in the venous blood draining are useful directional indicators of the state of oxygenation of the organ in question. The complexity of the arrangement has been elegantly illustrated by Intaglietta and colleagues in tissues which can be conveniently transilluminated for intravital microscopic observation and quantitative analysis.

Using the core equation of the oxygen transport system as defined above by Eq. 2.3:

$$\mathbf{DO_2} = \mathbf{Q} \times \mathbf{CO_2}$$

Of the two critical determinants of oxygen delivery, oxygen content is principally dependent on hemoglobin concentration [HGb]. The prevailing $pO_2$ determines the oxyhemoglobin saturation ($S_{O2}$) according to the ODC (According to the Hill equation:

$$\mathbf{SO_2} = \{(\mathbf{pO_2})^{\mathbf{n}}\} / \{(\mathbf{pO_2})^{\mathbf{n}} + (\mathbf{p_{50}})^{\mathbf{n}}\} \tag{2.6}$$

where n = 2.6, is the Hill coefficient).

At this time Eqs. 2.3 and 2.4 need to be expanded by the application of the *Fick principle* which states that the rate of oxygen entering the capillary is equal to the sum of the rate oxygen exiting and that consumed:

$$\mathbf{Q} \times \mathbf{CaO_2} = \{\mathbf{Q} \times \mathbf{CvO_2}\} + \mathbf{V_{O_2}} \tag{2.7}$$

And the oxygen content in Eq. 2.7 is:

$$\mathbf{CO_2} = \{\mathbf{SO_2} \times [\mathbf{HGb}] \times \mathbf{1.36}\} + \{\mathbf{pO_2} \times \mathbf{0.0031}\} \tag{2.8}$$

And Eq. 2.4 is expanded as:

$$\mathbf{E} = \{(\mathbf{CaO_2} - \mathbf{CvO_2})/\mathbf{CaO_2}\} \tag{2.9}$$

Thus, E, the extraction ratio, is the fraction of oxygen *extracted* from the arterial blood content, will yield the oxyhemoglobin saturation in the "venous" blood emerging from the microcirculation, and the corresponding $pO_2$ at the end of the capillary is the pressure driving diffusion to the cells. The $pO_2$ of blood entering and leaving the capillary and the rate of oxygen extraction en route determine the *mean tissue* $pO_2$ in a complex manner, and directional changes are reflected in the end-capillary $pO_2$.

Another way of analyzing the oxygen transport system is by the relationship of oxygen delivery to oxygen consumption (Leach and Treacher 1992).[2] The basis of the analysis is plotting oxygen consumption against oxygen delivery. Normally over a certain range of decreasing oxygen delivery, consumption is relatively independent of the delivery. Within this range demand may be satisfied by increased extraction of oxygen, a widening of the arterio-venous oxygen content difference, to an extent that no significant hypoxia is incurred. However, when delivery is reduced further a point of inflexion is reached and oxygen consumption declines nearly linearly, as delivery continues to decline. The point of inflexion represents the conditions that are such that a significant "mass" of cells becomes so hypoxic that it is no longer capable of using oxygen and a shift to anaerobic metabolism occurs. In this analysis the hypoxic cells unable to consume oxygen are not identified, only that they are sufficient in magnitude that their effect becomes observable. At this point some of the indicators of "hypoxia", or anaerobiosis can be observed (e.g. lactate production, etc.) (Leach and Treacher 1998; Schober and Schwarte 2012).

The consequences of pathological failure of the system to deliver sufficient oxygen for the prevailing metabolic needs are reduction in the tissue $pO_2$ or hypoxia(Leach and Treacher 1998). This may be incurred under the following circumstances:

1. Of the two components of Oxygen Delivery rate, blood flow can be varied but the blood hemoglobin concentration is normally fixed at approximately 140–160 g/L. Hence the complementary response to a content deficiency is to augment blood flow,

[2] This is an excellent introduction to most aspects of oxygen transport.

a. Significantly decreased [HGb] results in reduced oxygen content and delivery and may result in *hypoxia* because of increased extraction and reduced end-capillary and mean tissue $pO_2$ unless the blood flow is increased proportionally.
b. The arterial blood oxygen content will be reduced in the presence of reduced alveolar $pO_2$, physiologically significant shunting, mismatching of ventilation and perfusion, and even in the presence of normal extraction, end-capillary and mean tissue $pO_2$ will be reduced, unless compensated for by augmented blood flow.
c. The arterial blood oxygen content will also be reduced in the presence of reduced alveolar $pO_2$, unless compensated for by augmented blood flow
d. Conversely, significantly increased [HGb] results in an increase in oxygen content and delivery, but at the cost a marked rise in blood viscosity requiring augmented cardiac work.
e. In some *obligate aerobic* organs (the heart) normal levels of oxygen extraction are nearly complete and further increments of extraction are limiting so that

    i. marked increase in work and metabolic oxygen demand must be satisfied by a proportional increment in blood flow;
    ii. restriction in blood flow *and* marked reduction in [HGb] in the face of a rise in work load and metabolic rate inevitably lead to tissue hypoxia;
    iii. a physiologically significant change in the oxyhemoglobin dissociation characteristics of the blood (increased affinity, reduction in $p_{50}$), in combination with reduced [HGb] may induce tissue hypoxia and limit the ability to respond to increased demand;
    iv. conversely, markedly increased [HGb] in the face of increased work load and metabolic need may lead to tissue hypoxia because the associated rise in blood viscosity may limit the flow increment.

2. The ability to respond to increased *systemic* demands by increasing the cardiac output may be limited by tissue hypoxia in the heart initiating a *vicious circle* whereby the organ's ability to respond by increasing the cardiac output over the normal dynamic range of five-fold is limited in the presence of functional impairment and abbreviated contractile reserve. The limitations may be due to:

    a. impaired contractility or *systolic dysfunction* may arise from a number of causes, including

        i. impaired energy metabolism (Rosca and Hoppel 2010), such as in coronary vascular disease, mitochondrial dysfunction, loss of effectively functioning myocyte mass (infarcts), myocardial disease, valvular heart disease, ischemia–reperfusion and stunning,
        ii. excessive afterload as in established hypertension,
        iii. myocardial hypertrophy (Machackova, Barta et al. 2006), by extending diffusion distances in myocardium,
        iv. heart failure (Little and Applegate 1993).

b. impaired filling or *diastolic dysfunction* (Bateman, Sharpe et al. 2003; Periasamy and Janssen 2008), as in

   i. myocardial hypertrophy(Machackova, Barta et al. 2006),
   ii. myocardial fibrosis,
   iii. heart failure.

c. idiopathic or disease-related complex cardiomyopathies, including

   i. diabetic cardiomyopathy (Boudina and Abel 2010),
   ii. sepsis-induced cardiomyopathy (Romero-Bernejo, Ruiz-Bailen et al. 2011; Fernandes and Cesar de Assuncao 2012)
   iii. heart failure,
   iv. stress-related cardiomyopathy (Richard 2011)
   iv. endothelial dysfunction (Endeman and Schriffrin 2004; Ding and Triggle 2005; Feletou and Vanhoutte 2006).

#### 2.7.1.1 Cardiac Energetics and the Coronary Circulation

Because the heart is an obligate aerobic organ and because it plays a central role in responding to changing demands for oxygen by changing cardiac output over a five-fold range that require marked changes in its metabolic work, a brief consideration of the coronary circulation is of special interest.

The myocardium does work in generating tension and pressure during isovolumic systole and in shortening in ejection. These two factors and the level of contractility at which the work is performed determine the *myocardial oxygen consumption* (Crossman 2004).

#### 2.7.1.2 The Special Characteristics of the Coronary Circulation

The myocardium is an obligate aerobic organ and requires continual delivery of oxygen to meet its metabolic activity that supports its work. At rest it receives about 5 % of the cardiac output to support all of its functions and consumes about 10 % of the body's energy output. It can tolerate hypoxia for only short periods because its ATP and CP (creatine phosphate) reserves are very limited. Hypoxia rapidly results in cessation of contractile activity.

Because the left ventricle generates high intraventricular pressure the intramural arteries that supply the subendocardial layers are subject to compression from the high intra-ventricular pressure, and flow within them may be completely interrupted at the peak of systole. Hence, perfusion of the subendocardial myocardium is largely restricted to ventricular diastole when the perfusing pressure is the aortic diastolic blood pressure, but intra-ventricular pressure is very low. As a result the subendocardial layers are especially vulnerable to hypoxic injury if flow is inadequate.

The coronary circulation is distinct in that it has the highest extraction of oxygen with especially low coronary venous $pO_2$ and oxygen saturation; at rest, oxygen saturation is of the order of 40 % and $pO_2$ is 25−30 mm Hg in coronary venous blood. This corresponds to the widest arterio-venous oxygen content difference in the body of about 11−12 mL/dL (Sheppard and VanHoutte 1979). This is reflected in the fact that myocardial oxygen consumption is a greater fraction of the total than blood flow. This high extraction limits the scope of increasing the heart's own oxygen delivery by substantially increasing further its oxygen extraction. Yet, the myocardium is capable of increasing its work output over a five-fold range. This can occur even in the presence of variable arterial blood pressure, the perfusion pressure for coronary flow. Moreover, the changes in heart rate that are evoked when the cardiac output needs to respond to increased demands will abbreviate the time available for myocardial perfusion during each cardiac cycle. The adaptive mechanism accounting for this capacity is the ability to increase markedly coronary blood flow, the other component of delivery, as well as the microcirculatory anatomy, the rich capillary network and the wide distribution of mitochondria and the presence of myoglobin.

Coronary blood flow is *auto-regulated* in that over a physiologically relevant range of arterial pressures coronary flow is largely independent of the pressure. But when the coronary arterioles are maximally dilated, flow is linearly related to pressure. The coronary circulation is also auto-regulated in the sense that coronary flow is adjusted over a large range by changing the arteriolar resistance to meet the prevailing demand for oxygen by the myocytes. Hence, the resistance at the level of the arterioles is highly variable. At rest basal coronary tone is quite large but it can be decreased several-fold by *active vasodilation*. The difference at any given perfusing pressure between the basal blood flow and that which can be achieved by *maximal active vasodilation is the coronary vasodilator reserve*. The magnitude of this normal reserve is the principal reason that the heart can respond to widely varying metabolic demands by increasing its own blood supply.

The physiological significance of the coronary vasodilator reserve lies in the pathophysiology of coronary vascular disease. There is practically no resistance to flow in the normal *majo*r distributing coronary arteries; the major site of resistance is downstream at the level of the arterioles. It is their maximal dilation that determines the coronary reserve. However, if there are atherosclerotic or other pathological obstructions in the larger arteries, adequate basal coronary flow to meet basal demands can only be achieved by a proportional *reduction* in arteriolar resistance. This abbreviates the range available for dilation in case of *enhanced demand*. The consequence is that the maximal achievable flow is limited and delivery cannot meet the demand associated with e.g. high level of exercise. Moreover, atheromatous plaques represent areas of endothelial dysfunction with further impairment of the vasodilator response.

A significant contributing factor that permits the normal heart to respond to large demands is its microcirculatory anatomy. The ratio of myocytes to capillaries is about unity, thereby assuring that diffusion distances are short, largely determined by the myocytes' diameter. In case of enhanced demand extra capillaries

can be recruited, but the important facilitating effect is the richness of mitochondria spread widely throughout the myocyte (Jones 1986), and the presence of myoglobin, facilitating oxygen diffusion within the myocytes themselves. When the heart hypertrophies by enlargement of the myocytes, diffusion distances become longer and this may affect oxygen supply to the myocytes adversely.

#### 2.7.1.3 What Does a Mitochondrion Do?

The oxygen-consuming process in the mitochondrion is localized in the five sequential enzyme complexes embedded in the inner membrane, comprising the *mitochondrial respiratory chain* (Duchen 1999). Four of the five complexes provide reduced NADH transporting free electrons to the fifth complex, ATP synthase, where oxidative phosphorylation of ADP takes place. In the process oxygen is used to generate water and $CO_2$. Some of the oxygen is not completely oxidized and becomes the substrate for the generation of *reactive oxygen species* (ROS) at two of the membrane bound enzyme complexes (Gao, Laude et al. 2008). One such ROS is the superoxide anion ($O_2^{-\cdot}$). Normally there are a variety of powerful antioxidant defenses within the mitochondrion such that ROS production is effectively detoxified, although deficiencies of antioxidants are associated with ROS-mediated damage to the mitochondrial DNA (Gao, Laude et al. 2008; Rosca and Hoppel 2010).

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# Chapter 3
# Biochemistry of Hemoglobin

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Human hemoglobin A (Hb) is the main protein component of red blood cells, making up to 97 % of their dry content. Hb plays a crucial role in vertebrates, as it carries oxygen from the lungs to the tissues for their oxidative metabolism. Overall, the ~750 g of circulating Hb increase 70-fold the blood oxygen capacity of plasma, in which only a small amount of oxygen is dissolved. Recent findings indicate that Hb is also expressed at low concentrations in cells other than erythrocytes (Schelshorn et al. 2009), including neurons, macrophages, alveolar cells, and mesangial cells, where it likely plays a role as antioxidant. Erythrocyte Hb was recently proposed to be physiologically relevant in nitric oxide homeostasis and in reactivity with nitrite (Gladwin 2007; Gladwin et al. 2009). Here, we will focus on the structural and functional aspects of oxygen transport, their impact on the development of Hb-based oxygen carriers and on the selection of the most suitable Hb source.

## 3.1 Hemoglobin Structure

Hemoglobin is a tetramer composed of two identical $\alpha\beta$ dimers that self-assembly in a $\alpha_2\beta_2$ structure (Fig. 3.1a). The $\alpha$ and $\beta$ subunits consist of 141 and 146 residues, respectively, organized in 7 and 8 helices connected through non-helical segments. Both subunits belong to the globin superfamily, which share the so-called globin fold. This fold exhibits a pocket that strongly binds a heme moiety (Fig. 3.1b), allowing each tetramer to bind up to four molecules of oxygen. The heme group is

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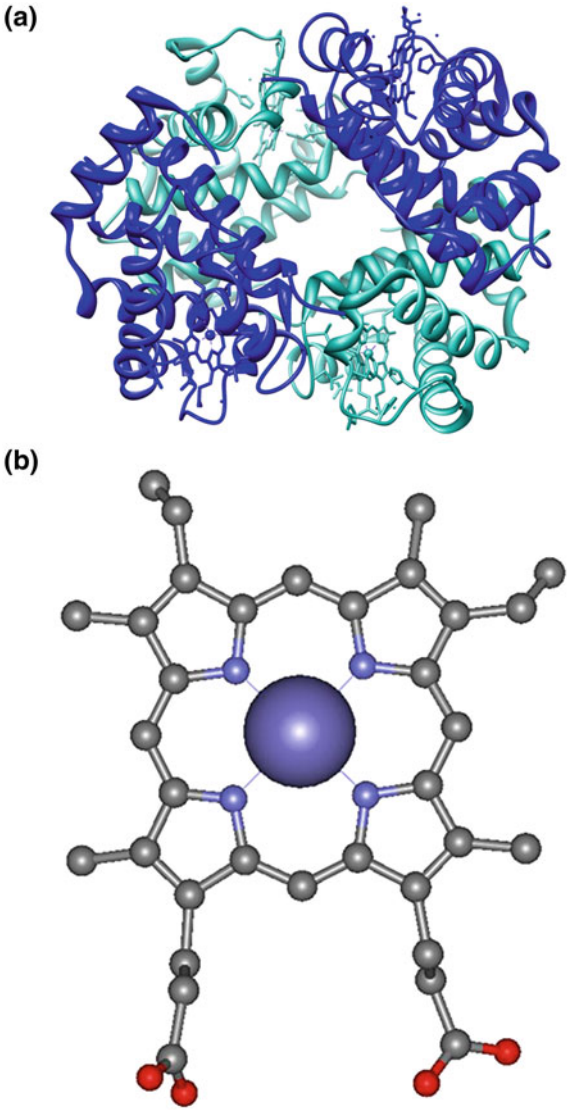


**Fig. 3.1** **a** Structure of T-state hemoglobin (pdb entry 2HHB). α subunits are in *light blue*, β subunits are in *dark blue*. **b** Heme moiety

located in a deep hydrophobic pocket formed by the E and F helices of each globin subunit. The heme iron, stabilized in the reduced form (+2) by the pocket environment, is coordinated by the four porphyrin nitrogens and the proximal histidine (F8) (Perutz 1970). On the distal side, the heme in the ferrous form can accept ligands such as molecular oxygen, carbon monoxide and nitrogen monoxide. In the ferric form, it binds cyanide and other ligands. Differences in the residues of the hydrophobic core formed by the A, B and E helices are responsible for the small differences between the α and β subunits in structure, dynamics and ligand binding.

Hb conformation depends on the presence of ligands of the heme moiety or effectors that bind at allosteric sites. Based on early data, the quaternary conformations crystallized as deoxygenated and fully ligated Hb were named T (tense) and R (relaxed) (Monod et al. 1965). It is now clear that the conformational space of Hb is more complex, including more R-like and T-like states (Dey et al. 2011). The main conformational changes in the T-R quaternary transition involve the intersubunit interfaces. In particular, upon ligation of the hemes, the $\alpha_1$ subunit shifts 6 Å with respect to the $\beta_2$ subunit, causing a 15° rotation and a 0.8 Å translation of one $\alpha\beta$ dimer with respect to the other. This conformational transition does not change significantly the $\alpha_1\beta_1$ interface, while the $\alpha_1\beta_2$ interface is markedly changed, with a narrowing of the central cavity where the allosteric effector 2,3-bisphosphoglicerate (2,3-BPG) binds. The complex modulation of the conformational equilibrium by both homotropic and heterotropic effectors makes Hb a paradigm of protein allostery and stimulated the development of several allosteric models to explain its behavior (Eaton et al. 1999; Koshland et al. 1966; Monod et al. 1965; Perutz 1970; Szabo and Karplus 1972; Viappiani et al. 2004).

## 3.2 Tetramer Stability

Hb tetramers can be described as tight $\alpha\beta$ heterodimers capable of forming looser $(\alpha\beta)_2$ tetramers, which constitute the largely prevalent form at physiological Hb concentrations inside the red blood cells. The relatively weak interactions at the $\alpha_1\beta_2$ interface allow for the conformational adjustments responsible for most aspects of the allosteric behavior of the tetramer, including the quaternary conformational changes associated with cooperativity. Because of the weaker interactions between the two $\alpha\beta$ dimers, Hb can undergo symmetrical reversible dissociation:

$$\alpha_2\beta_2 \rightleftarrows 2\alpha\beta$$

The corresponding equilibrium dissociation constant $K_D$ can be described as the ratio $K_D = [\text{dimer}]^2 \big/ \text{tetramer}$.

The tetramer dissociation is more pronounced in R state Hb, with a constant in the micromolar range at low ionic strength and neutral pH, as measured using a vast array of techniques (Antonini and Brunori 1971). Different estimates arise from the weakly distinguishable functional and spectroscopic properties of dimers with respect to tetramers. The dissociation constant was shown to depend significantly on the oxidation state of the heme, as well as pH, temperature and allosteric effectors (Antonini and Brunori 1971). As for T state Hb, significant dissociation occurs at far lower concentrations, with a $K_D$ in the order of $10^{-11}$ M. The formation of the complex of deoxy Hb with inositol hexaphosphate lowers the $K_D$ even further.

Overall, tetramer dissociation into dimers is negligible in the environment of the red blood cell, where Hb concentration is in the millimolar range. However, it has great relevance in the conditions where hemolysis leads to the presence of cell-free Hb in plasma, such as in hemolytic anemia. Some naturally occurring Hb mutants, as well as some decorated Hb-based oxygen carriers, show a higher degree of tetramer dissociation with respect to wild type Hb (Caccia et al. 2009). In vivo, dimers resulting from red cell lysis are sequestered by haptoglobin. At very low Hb concentrations, the dimers might undergo further dissociation into monomers, but the very low dissociation constant makes monomers unlikely to play any physiological role.

## 3.3 Hemoglobin Ligand Binding and Reactivity

In the deoxy state, iron(II) is five-coordinated and projects out of the porphyrin plane, pointing toward the proximal histidine. When a ligand binds at the distal side, it pulls the iron towards the plane of the porphyrin ring. Perutz (Perutz 1970) partly attributed the low oxygen affinity in the T state to a tension in the Fe–His(F8) bond, restraining the Fe from moving into the porphyrin plane. Upon ligand binding, the two $\alpha\beta$ dimers, arranged around a 2-fold axis of symmetry, rotate one respect to the other, causing the narrowing of the large central water cavity in the deoxy structure. The $\alpha$- and $\beta$-clefts define the two entry points into the central water cavity. The quaternary rearrangements triggered by ligation are similar for the three known gaseous ligands, $O_2$, CO and NO. However, due to differences in bond strength and orientation, there are also considerable differences. Specifically, the very strong affinity of NO to deoxyHb leads to the rupture of the bond between Fe–His(F8). Moreover, in addition to binding, both $O_2$ and NO chemically react with the heme iron.

### *3.3.1 Hemoglobin Reactivity with Oxygen*

#### 3.3.1.1 Oxygen Binding

DeoxyHb reversibly binds molecular oxygen ($O_2$):

$$\text{Heme-Fe(II)} + O_2 \rightleftarrows \text{Heme-Fe(II)-}O_2$$

The Hb oxygen dissociation curve shows a typical sigmoidal shape, as reported in Fig. 3.2, indicating a cooperative oxygen binding. The cooperativity assures that a proper amount of oxygen is released in the range between 100 torr of oxygen tension ($pO_2$) in the lungs and 20 torr $pO_2$ in the tissues. As a matter of fact, when red blood cells are in the lungs, Hb is 97 % saturated with oxygen. At the capillary

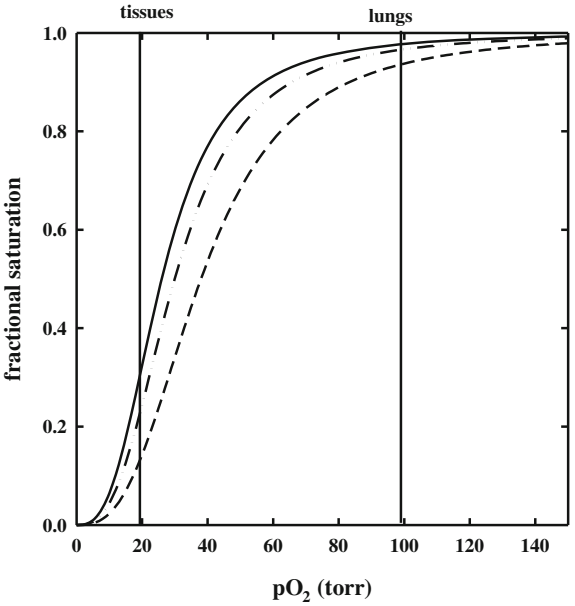


**Fig. 3.2** Oxygen binding curve of human hemoglobin at pH 7.4, in the absence of $CO_2$ (*solid line*), at pH 7.2 in the absence of $CO_2$ (*dash-dot-dot line*), and at pH 7.2 in the presence of 40 torr of $CO_2$ (*dash line*). Considering a typical partial pressure in the tissues of 20 torr of oxygen, hemoglobin in the three conditions unloads 65, 73 and 80 % respectively

level in the tissues, saturation drops to 30 %. Myoglobin, a typically non-cooperative oxygen binding proteins, would be 98 % saturated in the lungs and 93 % saturated in the tissue.

The affinity for Hb for oxygen is commonly expressed in terms of $P_{50}$, i.e. the oxygen tension at which half of the heme site are saturated with oxygen. For human blood, at physiological pH (7.4) and temperature (37 °C), the $P_{50}$ is around 26 torr. The oxygen binding curves can be reported using the Hill equation:

$$\log \frac{Y}{1-Y} = n \log \left( \frac{pO^2}{p50} \right)$$

The corresponding plot is linear between fractional saturations of 0.1 and 0.9. The slope (n), or Hill coefficient, is a measure of the cooperativity of the binding. This number does not possess a physical meaning and can be lower than 1 (negative cooperativity), 1 (non-cooperative binding), higher than 1 (positive cooperativity). For Hb, its value is between 2.8 and 3.

### 3.3.1.2 Hemoglobin Autooxidation

Hb undergoes a slow spontaneous auto-oxidation in the presence of molecular oxygen, producing the highly reactive superoxide anion and a functionally inactive Fe(III) heme, which immediately binds either a water molecule or a hydroxide anion:

$$\text{Heme-Fe(II)-O}_2 \rightleftarrows \text{Heme-Fe(III)} + \text{O}_2^-$$

An aquo-met heme in a partially oxidized tetramer alters the conformational equilibrium, preventing the full transition to the T state and shifting the dissociation curve towards higher affinities. The overall effect is a decrease in oxygen delivery due both to the loss of active hemes able to bind oxygen and to the increased affinity of partially oxidized Hb tetramers. The oxidation rate is also increased in Hb dimers with respect to tetramers. Every day, around 3 % of circulating Hb undergoes autooxidation, but the complex redox enzymatic machinery inside the red blood cells keeps it below 1 %. In particular, the autooxidation of Hb is counteracted by met-Hb reductases. Its action is crucial because met-Hb tends to release the Fe(III) heme group, that, being hydrophobic, can bind the lipid membrane and accelerate the degradation of the red blood cells.

### 3.3.2 Hemoglobin Reactivity with Carbon Monoxide

Carbon monoxide is commonly known as a toxic gas molecule due to its strong binding to Hb:

$$\text{Heme-Fe(II)} + \text{CO} \rightleftarrows \text{Heme-Fe(II)}-\text{CO}$$

The affinity of carbon monoxide for Hb is about 250-fold higher than that of oxygen. Therefore, exposure to carbon monoxide causes oxygen displacement and leads to a hypoxic state that may cause death. Recently, however, this gas gained interest as its beneficial effects at very low concentration emerged in various in vitro and in vivo experiments (Bösch and Tsui 2012). In fact, directly exposure of carbon monoxide may protect cells or organs from various disease insults, due to its anti-inflammatory, anti-apoptotic and anti-proliferative properties (Loop et al. 2012). The Hb-based oxygen carrier MP4 (Hemospan®), a polyethyleneglycol (PEG)-conjugated Hb undergoing clinical trials as an oxygen carrier, is also studied as a CO delivery agent, and was shown to reduce the infarct size when administered prior to the induction of ischaemia in rat models (Vandegriff et al. 2008). These recent experimental evidences highlight the fact that the effects of carbon monoxide can greatly vary depending on the amount.

### 3.3.3 Hemoglobin Reactivity with Nitric Oxide

Nitric oxide, or nitrogen monoxide (NO), is a free radical that can stabilize its unpaired electron by reacting with species containing other unpaired electrons or by interacting with the *d*-orbitals of transition metals, particularly iron. NO can reversibly bind the heme group of both Fe(III) and Fe(II) Hb. However, it can also react through a complicated redox chemistry with the heme iron, other heme

ligands and cysteine residues (Gow and Stamler 1998; McMahon et al. 2002). The combination and the competition of these reactions, the high reaction rates of some of them and the inequivalence of the $\alpha$ and $\beta$ subunits have long delayed the full understanding of the interactions between NO and Hb. However, considering the crucial role of NO as a biomediator, its interactions with Hb might play a physiologically crucial role in the regulation of several signaling pathways, particularly those responsible for the modulation of vasoactivity.

#### 3.3.3.1 Binding of Nitric Oxide to the Heme Iron

Nitric oxide binds the ferrous heme iron with a nearly diffusion-limited rate ($\sim 10^7 M^{-1} S^{-1}$) (Olson et al. 2004), forming (iron-) nitrosyl-Hb (NO–Hb):

$$\text{Heme-Fe(II)} + \text{NO} \rightleftarrows \text{Heme-Fe(II)}-\text{NO}$$

The dissociation of NO from fully nitrosylated (R state) Hb is extremely slow, with a $t_{1/2}$ in the order of hours, making the competition with $O_2$ and CO virtually negligible. The bond of NO with the $\alpha$ heme iron is much stronger than that of $O_2$, causing the detachment of the proximal histidine of the $\alpha$ subunits, as revealed by a characteristic hyperfine splitting in EPR spectra. The resulting pentacoordinated $\alpha$-Fe(II)-NO is stable, with a dissociation rate constant in the order of years. As the displacement of the proximal histidine upon oxygen ligation is crucial in the mechanism of cooperativity, it is not surprising that the binding of NO to deoxyHb, unlike that of $O_2$ and CO, occurs in a non-cooperative fashion. Despite partial pentacoordination, fully nitrosylated Hb is in an R quaternary state.

NO dissociation is much faster in partially nitrosylated (T state) tetramers, a condition that is much more likely to occur physiologically, especially in venous blood. Under these conditions, a transfer of NO from the $\beta$ chains to the $\alpha$ chains was observed, resulting in a slow increase in overall pentacoordination:

$$\begin{aligned}&(\beta-)\text{Heme-Fe(II)}-\text{NO} + (\alpha-)\text{Heme-Fe(II)}\\ &\quad\rightarrow (\beta-)\text{Heme-Fe(II)} + (\alpha-)\text{Heme-Fe(II)}-\text{NO}\end{aligned}$$

Unlike CO and $O_2$, NO also binds to the ferric form of Hb. However, the Fe(III)-NO complex tends to give a charge transfer, reducing the iron to the ferrous form and producing the highly reactive nitrosonium ion ($NO^+$):

$$\begin{aligned}\text{Heme-Fe(III)}-\text{NO} &\rightleftarrows \text{Heme-Fe(II)}-\text{NO}^+\\ \text{Heme-Fe(II)}-\text{NO}^+ + \text{H}_2\text{O} &\rightleftarrows \text{Heme-Fe(II)} + \text{NO}_2^- + 2\text{H}^+\end{aligned}$$

The attack of either a water molecule or a hydroxide ion to the ferrous nitrosonium ion complex results in the auto-reduction of the heme and in the release of a nitrite ion:

$$\text{Heme-Fe(II)}-\text{NO}^+ + \text{H}_2\text{O} \rightleftarrows \text{Heme-Fe(II)} + \text{NO}_2^- + 2\text{H}^+$$

#### 3.3.3.2 Nitrite Reductase Reactivity

In the reverse reaction of nitrite formation, deoxyHb reacts with nitrite ions producing nitric oxide and Fe(III) hemes:

$$\text{Heme-Fe(II)} + NO_2^- + 2H^+ \rightleftarrows \text{Heme-Fe(II)}{-}NO^+ + H_2O \rightleftarrows \text{Heme-Fe(III)} + NO$$

The resulting NO molecules quickly bind deoxyHb, producing nitrosyl-hemoglobin. Nitrites, therefore, represent an important biological reservoir of NO and the reaction was suggested to contribute to NO bioavailability. R-state hemes and T-state hemes react with nitrites with very different rates, with the bimolecular rate of the reaction between nitrite and R-state Hb being much higher $\left(6\ M^{-1}s^{-1}\right)$ than that of T-state Hb $\left(0.03\ M^{-1}s^{-1}\right)$. Considering that both products of the reaction, Fe(III) hemes and nitrosylated hemes, tend to stabilize the R state, nitrite reduction is an autocatalytic reaction. Moreover, the apparent rate depends on the presence and concentration of allosteric effectors capable of modulating the quaternary conformational equilibrium, such as inositol hexaphosphate, protons and oxygen. In vivo, the reaction is likely relevant in hypoxic red blood cells, where unliganded subunits are available. Hb can also catalyze the conversion of two nitrite ions to dinitrogen trioxide $(N_2O_3)$, an intermediate that concentrates in the hydrophobic membranes of red blood cells and that can either dissociate in NO or form nitrosate thiols, two suggested pathways for NO escape from erythrocytes.

#### 3.3.3.3 S-nitrosyilation

NO ligated to ferric heme was shown to be transferred to the thiol of cysteine $\beta$93 (Gow and Stamler 1998; Jia et al. 1996; Stamler et al. 1997):

$$\text{Heme-Fe(III)}{-}NO + \text{Cys-S}^- \rightleftarrows \text{Heme-Fe(II)} + \text{Cys-SNO}$$

The nitrosothiol product can be rationalized as the formal $NO^+$ transfer to a protein thiolate instead of water, as it is the case of nitrate formation. Either pathways lead to the production of Fe(II) hemes, which are immediately converted to Fe(II)NO. Human adult Hb has six cysteines, two in the $\alpha$ chains and four in the $\beta$ chains. However, only the highly conserved $\beta$93 cysteine becomes *S*-nitrosylated. The unique position of this residue at the interdimer interface explains the allosteric effects associated with its nitrosylation: SNO formation is favored in oxygenated Hb over deoxygenated Hb, the stability of SNO-Hb is significantly greater for the R state molecule than the T-state molecule and S-nitrosylation of this cysteine residue has a positive allosteric effect on oxygen binding. It was proposed that SNO-Hb is formed in the lungs, where Hb is in the R (oxy) state, while the allosteric transition to the T (deoxy) state during the arterial-venous transit favours NO release and thus promotes vasorelaxation in tissues with low $pO_2$ (Gow and Stamler 1998).

As NO cannot be directly transferred from red blood cell-confined Hb to its cellular targets in the vasculature, a transnitrosation reaction to cysteine residues of other proteins or to low molecular weight thiols has been proposed as a delivery pathway:

$$\text{Hb-SNO} + \text{R1-S}^- + \text{H2O} \rightleftarrows \text{Hb-S}^- + \text{R1-SNO}$$

S-nitrosothiols have a significantly longer half-life than free NO and are not scavenged by heme. Therefore, through this mechanism, NO can be transported in the bloodstream, channeled in a protected form and then released in the proximity of its cellular targets (Allen et al. 2009). This hypothesis has been recently challenged based both on the evaluation of NO chemistry with hemoglobin (Xu et al. 2003) and on the apparent negligible effect of cysteine $\beta 93$ on vasoactivity in knock-out mice (Isbell et al. 2008).

#### 3.3.3.4 NO Dioxygenase Reactivity

NO rapidly reacts with oxyHb, producing ferric heme and nitrate (Doyle and Hoekstra 1981):

$$\text{Heme-Fe(II)}-\text{O}_2 + \text{NO} \rightarrow \text{Heme-Fe(III)} + \text{NO}_3^-$$

The reaction rate was determined to be $4 \times 10^7\ \text{s}^{-1}\text{M}^{-1}$, in the same order of magnitude of NO binding to unliganded hemes. The reaction mechanism can be better explained assuming the ionic character (i.e. ferric superoxide) of the Fe(II)–$O_2$ complex. The interaction with NO transiently produces a peroxynitrite intermediate:

$$\text{Heme-Fe(III)}-\text{O}_2^{\cdot-} + \text{NO} \rightarrow \text{Heme-Fe(III)}-\text{ONOO}^- \rightarrow \text{Heme-Fe(III)} + \text{NO}_3^-$$

It has been suggested that this reaction is responsible for the rapid removal of NO from the vasculature. As a matter of fact, the half-life of NO is around 1 min in plasma, but only in the millisecond range in the presence of red blood cells. Still, the removal of NO by red blood cells is estimated to be 600–1000-fold slower than that carried out by free Hb, due to the diffusional barriers offered by the red cells membrane.

## 3.4 Allosteric Effectors of Hemoglobin

Several allosteric effectors, i.e. molecules that bind at sites other than heme, can modulate the equilibrium between high oxygen affinity forms and low oxygen affinity forms, often in a synergistic or antagonistic way (Imai 1982). The presence of the physiological effectors, the concentration of which can change in the bloodstream, allows Hb to be optimally modulated for oxygen delivery. The allosteric equilibrium of Hb is controlled by effectors that shift the equilibrium

either towards the T state or the R state. The stabilization of the relaxed state shifts the oxygen equilibrium curve to the left, producing a high affinity Hb that more readily binds and holds oxygen. A shift toward the T state produces a low affinity Hb that readily releases oxygen. The degree of shift in the oxygen binding curve is reported as an increase or decrease in $P_{50}$.

### 3.4.1 Bohr Effect

The heterotropic effect exerted by protons on oxygen affinity has emerged early in the investigations of Hb and was named Bohr effect (Bohr et al. 1904). More specifically, the Bohr effect in the pH range 6–9 was named alkaline Bohr effect, and consists in an increase in oxygen affinity as the proton activity decreases. The acid, or reversed Bohr effect, takes place below pH 6, where the oxygen affinity rises as the pH decreases. Only the alkaline Bohr effect is thought to have physiological relevance, both in buffering part the protons produced by the metabolic formation of carbonic acid in the tissues and in enhancing the unloading capacity of oxygen at the level of venous blood, where the pH is slightly lower with respect to oxygenated blood (7.2 vs. 7.4).

From a molecular point of view, the alkaline Bohr effect is associated with the protonation/deprotonation of amino acid residues involved in the interdimeric interface and in the $\beta$ subunit (the Bohr groups), thus affecting the relative stability of the T and R states. Oxygenation decreases the $pK_a$ of the Bohr groups (Hb becomes more acidic) and protons are released:

$$\text{H-Hb} + \text{O}_2 \rightleftarrows \text{Hb-O}_2 + \text{H}^+$$

The $\alpha$-amino groups of the amino terminal residues of the $\alpha$ chains and the histidine residues $\beta$146 and $\alpha$122 are known as Bohr groups. In particular, the carboxy terminal histidines of the $\beta$ chains account for more than 50 % of the Bohr effect. It has to be noticed that other ionic allosteric effectors, such as 2,3-BPG and chloride ions, can alter the $pK_a$ of some amino acid side chains by forming ionic bonds with them. In particular, the presence of organic phosphates at physiological concentrations increases the Bohr effect further.

### 3.4.2 $CO_2$

$CO_2$ reacts with the N-terminal amino groups of the chains of Hb to form carbaminohemoglobin:

$$\text{R-NH} + \text{CO}_2 \rightleftarrows \text{R-NH-CO}_2^- + \text{H}^+$$

The newly formed negative charge can take part in intersubunit salt bridges that stabilize the T state, thus contributing to the release of more oxygen to the venous

blood, where $CO_2$ if formed during the oxidative metabolism. Overall, the decrease in pH and the direct allosteric effect of $CO_2$ are synergistically responsible for a marked increase in oxygen off-loading in the venous system (Fig. 3.2).

### *3.4.3 Organic Phosphates*

The first reported allosteric effector of human Hb was the natural effector, 2,3-BPG, which forms a stoichiometric complex with Hb. The structure of deoxygenated Hb in complex with 2,3-BPG shows the effector binding at the $\beta$-cleft (on the two fold axis) via salt bridges that tie the two $\beta$-subunits together, making it difficult for the T-R transition to take place. Even though 2,3-BPG is known to bind liganded Hb, the significantly smaller $\beta$-cleft precludes an equally strong interaction. The preferential binding of 2,3-BPG to deoxygenated Hb stabilizes the T state relative to the R state and decreases the affinity of Hb for oxygen, inducing a right shift of the Hb oxygen binding curve, with the $P_{50}$ increasing from 12 to 26 torr at physiological temperature and pH. 2,3-BPG modulates the intrinsic affinity of the T and R quaternary states and affects the tetramer stability in both conformations. It also binds free $\alpha\beta$ dimers.

A number of synthetic effectors have been found to bind at the $\alpha$- or $\beta$-cleft or at the middle of the central water cavity and to shift the oxygen binding curve either to the left or to the right (Abraham et al. 1995). These compounds are long recognized as potential therapeutic agents for the treatment of a variety of conditions for which a transient increase in oxygen delivery to tissues or increase in the oxygen affinity of Hb is beneficial. Among the structural analogs of 2,3-BPG, inositol hexaphosphate (IHP) has been extensively used in the investigation of the allosteric properties of Hb because of the strength of its complex, being 1000 times more stable than that with 2,3-BPG.

In the early 1980s, a new group of right shifters of the oxygen binding curve was recognized by investigating the interaction between Hb and two antilipidemic drugs, bezafibrate and clofibrate. These molecules bind Hb in a 2:1 complex at two symmetry-related binding sites located in the central water cavity of deoxyHb, 20 Å apart from the binding site of 2,3-BPG and its analogues. Similarly to 2,3-BPG, they stabilize the low affinity conformations, thus reducing the overall affinity of Hb for oxygen. They act synergistically with the physiologically available 2,3-BPG, as they bind to a different site.

## 3.5 Hemoglobin-Based Oxygen Carriers

The deep knowledge of Hbs is a crucial requirement for the development of Hb-based oxygen carriers as potential hemoglobin-based blood substitutes (HBOCs). These products might be potentially capable of overcoming some issues associated

with blood transfusions, such as cross-matching and blood typing, limitations in the availability of healthy donors, a short shelf life and concerns about contamination by infectious agents. The need to obtain a cell-free circulating molecule that binds oxygen with properties close to that of hemoglobin within the red blood cell has led to the investigation of hemoglobins from a number of sources, including humans, animals and recombinant proteins.

### *3.5.1 Human Adult Hb*

Human hemoglobin from outdated blood is an obvious source for HBOCs, as it available in a relatively large quantity. Usually, about 5 % of blood units are discarded either for technical reasons or aging. The idea of substituting whole blood with purified free human Hb emerged at the end of nineteenth century. However, the infusion of Hb solutions initially showed several toxic effects, mainly on kidneys and the cardiovascular system. These effects have been widely studied and, although most of them are still under investigation, in many cases their biochemical and physiological basis are known. One of the critical points is the dimerization of Hb tetramers occurring at concentrations lower than those inside the erythrocytes. As a matter of fact, as Hb dimers are sufficiently small to be filtered at the glomerular level, they tend to cause nephrotoxicity. Moreover, the reducing agents present in the plasma are not as effective as the enzymatic systems found inside the erythrocytes and the oxidation process leads to the formation not only of met-Hb, unable to bind oxygen, but also of reactive oxygen species such as the superoxide anion, $H_2O_2$, ferryl hemes and heme degradation products. The Hb molecule, without the protection of the red blood cell envelope, can also cross the walls of blood vessels, exerting a NO scavenging activity both in the lumen and in the interstitial space, causing vasoconstriction. Chemical modifications have been explored to overcome the toxic effects of free hemoglobin. They include intra- and inter-tetramer cross-linking, polymerization, surface decoration with dextran, polyethylene glycol (PEG) or polyoxyethylene and physical immobilization either in polymeric matrices, or within nanocapsules or lipid vesicles, or onto the surface of nanoparticles.

### *3.5.2 Fetal Hb*

Fetal Hb (HbF) has been proposed as an alternative to adult Hb for the preparation of HBOCs. The rationale behind this choice is that HbF has a high affinity and therefore can transport a larger amount of oxygen. The disadvantage is that a high affinity Hb might not be able to release oxygen in the tissues. It is well known that the high affinity of HbF is predominantly due to the single amino acid mutation at residue 143. The substitution of His at the $\alpha$ chain with Ser leads to a lower affinity

of 2,3 BPG to Hb, thus to an increase in oxygen affinity. At 4 °C, pH 7.2, 1 mM 2,3BPG, HbA exhibits a $P_{50}$ of about 4.2 torr, whereas HbF exhibits a $P_{50}$ of about 2.8 torr (Bunn and Forget 1986). As the cord blood contains about 80 % of HbF, transfusions using cord blood have been applied as a blood substitute for emergency patients in Third World due to its sterile conditions (Bhattacharya 2005). However, the overall available quantity of HbF is low, thus hampering its use in the preparation of HBOCs.

### *3.5.3 Bovine Hb*

The requirement of a large supply of Hb to be used in the development of a safe and effective HBOC led to search for alternative sources with respect to outdated human blood. Bovine Hb represents an attractive alternative to human Hb due to the possibility of recovering significant quantity of Hb from animals sacrificed in the meat industry. The key feature of bovine Hb is the almost full insensitivity to 2,3 BPG. This feature is shared by sheep, cat and goat Hbs. Moreover, the 2,3 BPG concentration within the red cell of these animals is lower (0.7 mM) than in humans (about 5 mM) (Bunn and Forget 1986). The main allosteric effector of bovine Hb is chloride, and, to a less extent, carbon dioxide. Therefore, bovine Hb free in the plasma undergoes oxygen affinity regulation by the synergic action of plasma chloride ions and carbon dioxide. A product consisting of bovine Hb decorated with 10-12 units of 5000 Da-MW PEG was investigated as a possible blood substitute and showed a $P_{50}$ of 10.2 torr at 37 °C, higher than that of PEGylated human Hb but still far from that of human blood (around 26 torr).

### *3.5.4 Antarctic Fish Hb*

In view of obtaining a HBOC with an intrinsically low $P_{50}$ in a cell-free environment, Hbs from the Antarctic fish Notothenioidei, the dominant suborder of teleosts in Antarctica, are particularly interesting. The oxygen affinity of these Hbs is exceptionally low (di Prisco et al. 2007), as an evolutionary adaptation to the high oxygen concentrations in the cold Antarctic waters. Moreover, unlike HbA, fish Hbs tetramers do not significantly dissociates into dimers, even in the ligated form (Giangiacomo et al. 2001). Finally, Cys $\beta$93, present in the great majority of vertebrate Hbs and known to perturb the properties of PEGylated Hbs and to scavenge NO (Caccia et al. 2009) is missing in Hbs of almost all teleosts, making them suitable for PEG decoration. Giving these favorable properties, the tetrameric Hb from *Trematomus bernacchii* (TbHb) was decorated with PEG with a protocol already used for human PEGylated Hb (Portoro et al. 2008). PEGylated TbHb, at pH 7 and 10 °C, showed a $P_{50}$ of $19.7 \pm 0.3$ torr, near the $P_{50}$ of human Hb in RBCs (Coppola et al. 2011).

### 3.5.5 Lumbricus Terrestris Erythrocruorin

*Lumbricus terrestris* (earthworm) erythrocruorin is an extracellular Hb with high molecular weight (3.6 MDa), low autoxidation rate, limited reactivity towards nitric oxide (NO) and a $P_{50}$ very similar to human RBCs inside red blood cells (28 torr at 37 °C). These properties make this oxygen carrier a potential starting material to be used as a blood substitute. Erythrocruorin was safely transfused into mice, rats, and hamsters without showing major side effects. Microvascular experiments demonstrated its capacity to deliver more oxygen than conventional plasma expanders (Elmer et al. 2012a). Erythrocruorin purified from earthworms was also evaluated in hamsters, where it did not elicit side effects such as hypertension or vasoconstriction (Elmer et al. 2012b).

### 3.5.6 Hemerytrin

Hemerythrin was first described in marine invertebrates (e.g., sipunculids, priapulids, brachiopods), as an example of oxygen binding protein with a non-heme di-iron binding site (Farmer et al. 2000; Jin et al. 2002) (Kryatov et al. 2005). It has been proposed for use as a blood substitutes since it avoids stress-related side reactions of heme proteins. As a matter of fact, oxy-hemerythrin does not react directly with nitric oxide and deoxy-hemerythrin binds NO with much lower affinity with respect to Hb. Moreover, the oxidized form does not cause formation of ferryl or free radicals (Kryatov et al. 2005; Mot et al. 2010). A chemical modification of the octameric form of hemerythrin from *Phascolopsis gouldii* with PEG and glutaraldehyde was carried out, resulting in derivatives with $P_{50}$ s spanning from 24- to 40 torr in PEGylated derivatives and from 6 to 17 in glutaraldehyde cross-linked derivatives (Mot et al. 2010). The safety of native and modified hemerythrin was compared to native and glutaraldehyde-polymerized bovine Hb on human leukocytes and umbilical vein endothelial cells, concluding that hemerythrin and its chemical derivatives are less toxic than bovine Hb (Fischer-Fodor et al. 2011).

### 3.5.7 Recombinant Hemoglobins

Since the discovery of hemoglobin S as the variant form responsible for sickle cell anemia, several hundred naturally occurring hemoglobin mutants have been identified. Many of them are clinically silent, but others significantly alter the functional properties of hemoglobin, particularly in terms of oxygen affinity, cooperativity, NO deoxygenase reactivity, rate of autoxidation, stability of the tetramer and capability to undergo autopolymerization. The wealth of information

gathered in these studies, followed by the characterization of point-mutated recombinant Hbs, allowed to investigate the specific role of individual amino acids in the regulation of the functional properties of hemoglobin and produce engineered variants showing a large variability in functional properties (Varnado et al. 2013). Although potentially more flexible than chemically modified Hb, recombinant Hb-based oxygen carriers are limited by the expression yield and by the purification procedures. Several systems for Hb heterologous expression are currently being developed, including bacterial expression, expression in transgenic animals and plants and in vitro differentiation of embryonic cells to hematopoietic stem cells. For example, human Hb was expressed in transgenic mice (Behringer et al. 1989) or in transgenic swine (Sharma and Gulati 1994; Swanson et al. 1992).

Examples of recombinant hemoglobins are reported below:

(1) *rHb0.1* It was produced by Somatogen and includes two fused α chains separated by a glycine linker, plus a mutation (N108 K) found in the naturally occurring Presbyterian Hb, endowed with a lower oxygen affinity and low cooperativity even in the absence of BPG. The α fusion was designed to prevent dimer dissociation, and was shown not to perturb either the quaternary or tertiary structures of rHb1.1 (Brucker 2000).

(2) *rHb1.1* (*Optro*®) The recombinant hemoglobin named rHb1.1 (Optro®) and originally developed at Somatogen in collaboration with the Laboratory of Molecular Biology in Cambridge (Brucker 2000; Loeb et al. 1997; Looker et al. 1992; Rattan et al. 1995), is based on rHb0.1. A further Val1 → Met mutation was introduced to optimize protein expression in bacteria. rHb1.1 was proposed as a blood substitute in elective surgery and as a stimulating agent of erythropoiesis. It was demonstrated that bone marrow depression following administration of AZT to normal and to murine models of AIDS is alleviated by the administration of rHb1.1 alone or in combination with erythropoietin (Moqattash et al. 1997). Furthermore, it was proposed that rHb1.1-induced renal vasoconstriction leads to the production of erythropoietin, thereby stimulating erythropoiesis, and that the administration of rHb1.1 together with erythropoietin can increase the production of RBCs (Chang 1997).

(3) *rHb2.0* Developed by Baxter Hemoglobin therapeutics (formerly Somatogen), it is based on rHb1.1 but contains additional mutations to reduce the NO dioxygenase activity. Studies with sperm whale myoglobin and human Hb showed that substitution of some residues in the distal heme pocket, namely LeuB10 and ValE11, can reduce the reaction rate of NO with oxyHb due to steric hindrance (Doherty et al. 1998; Eich et al. 1996; Hartman et al. 1998; Olson et al. 2004). In a subsequent work (Doherty et al. 1998), a series of mutant recombinant di-α-Hbs was produced by changing on the $\beta$ chains the residues LeuB10, ValE11 and LeuG8, located in the distal heme pocket, to larger hydrophobic residues (Phe, Leu, Trp and Ile). As a result, changes in both oxygen affinity and rate of NO reaction were obtained. This study demonstrated for the first time that NO scavenging is directly correlated with

NO-dependent oxidation rate of oxyHb and that the rate of NO scavenging correlates with the pressure responses observed upon administration of recombinant Hb. rHb2.0 exhibits a 20 to 30 times lower NO-scavenging rate with respect to Hb and rHb1.1.

(4) *rHb3011* A recombinant Hb (rHb3011) with a reduced rate of NO scavenging and decreased oxygen affinity was shown to induce a reduced gastrointestinal dysmotility, measured as percent emptying of the stomach, in comparison with rHb1.1.

(5) *Hemoglobin Polytaur* It is an auto-polymerizing Hb mutant designed via site directed mutagenesis following the observation that an additional surface Cys residue in the natural mutant hemoglobin Porto Alegre ($\beta$Ser9 → Cys) induces polymerization through the formation of inter-chain disulfide bonds (Baudin-Creuza et al. 2002; Tondo et al. 1974). Hb Polytaur is a recombinant $\alpha$-human/$\beta$-bovine hybrid hemoglobin capable of forming inter-tetramer disulfide bridges due to the $\beta$Ser9 → Cys mutation, promoting spontaneous inter-tetramer polymerization (Bobofchak et al. 2003; Faggiano et al. 2011; Fronticelli and Koehler 2009; Fronticelli et al. 2007). Being based on bovine Hb, it shows an intrinsic lower affinity for oxygen with respect to human hemoglobin and is not regulated by BPG, which is not present in bovine erythrocytes.

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# Chapter 4
# The Role of Blood and Plasma Viscosity in Restoring Oxygen Delivery Capacity

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## 4.1 Introduction

It is a generalized perception that blood products are needed when oxygen ($O_2$) delivery capacity is jeopardized by the decrease of blood's intrinsic $O_2$ carrying capacity due to the decrease of hematocrit (Hct) or blood hemoglobin. Using a blood transfusion is certainly a direct, proven and in principle effective way of remedying this problem, and since a blood transfusion restores blood volume and $O_2$ carrying capacity it is logical that the development of blood substitutes should focus on this goal.

Advances in recombinant technology and stem cell manipulation may produce a blood substitute with most of the features of blood, including encapsulation of the $O_2$ carrying agent at high concentration within a membrane, i.e., a red blood cell (RBC) like $O_2$ carrier. However production of clinical materials in quantities

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sufficient for blood transfusion scenarios is unlikely to become available at costs within means of most health care systems. This situation restricts the development of blood substitutes to hemoglobin (Hb) solutions and vesicle encapsulated Hb suspensions (HBOCs, hemoglobin based oxygen carriers) with physical properties that are significantly different from blood.

It is well established that biological systems are sensitive to mechanical stimuli such as hydraulic pressure and shear stress, and react to their action by stimulating production of a host of chemicals, cytokines and genes, a process labeled biochemical mechanotransduction. One of the most important mechanical interactions which occur in the circulation is at the blood tissue interface which comprises the edge of the blood column, the cell free layer, the glycocalyx and the endothelium. In this region the interaction between blood and the vessel wall is fundamentally affected by the composition of blood and the resulting flow properties, which ultimately determines the functionality of the microcirculation, therefore the changes in blood physical and flow properties induced by HBOCs can play a significant role in the process of blood substitution.

## 4.2 Maintenance of Microvascular Function: A Necessary HBOC Property

The restoration of blood's intrinsic $O_2$ carrying capacity is ineffectual unless the microcirculation is functional; therefore a fluid that replaces blood and carries $O_2$ will not achieve its purpose unless simultaneously maintaining microvascular function.

Remarkably most intravenous fluid i.e., plasma expanders, administered in critical illness and in the treatment for blood losses were primarily designed for blood volume maintenance, a role in which they are used up to the so called "transfusion trigger". This point defines the condition at which a blood transfusion is indicated and fluid volume maintenance by plasma expanders in combination with the remaining $O_2$ carrying capacity of RBCs is estimated to have exhausted its ability to maintain tissue metabolism. However, the need to attain a functional microcirculation is not explicitly addressed in the formulation of presently used plasma expanders.

The principal properties that characterize plasma expanders are the molecular nature of the solute, period of intravascular retention, coagulation related factors and cost. The use of crystalloids leads to tissue edema, which is avoided by the use of colloids. Administering a plasma expander up to the transfusion trigger is a necessary and ubiquitous component of treatment and resuscitation; however objectives beyond the normalization of blood volume are not explicitly identified or addressed.

Experimental studies in the field of shock resuscitation highlight the role of microvascular function in resuscitation (Salazar Vázquez et al. 2008), showing that the critical issue in resuscitation is maintenance of microvascular function and restoration of $O_2$ carrying capacity which are reduced in parallel during

hemorrhage, hypovolemia, etc. Notably restoration of $O_2$ carrying capacity *per se* is not necessarily sufficient to treat blood losses unless microvascular function is restored. Conversely, restoration or maintenance of microvascular function even during significant decreases of intrinsic $O_2$ carrying capacity can compensate for the reduced $O_2$ transport capacity, since comparatively few RBCs suffice to oxygenate the tissue and maintain metabolism, if microvascular function is normal. These considerations are important in designing of HBOCs and formulating their $O_2$ carrying capacity, which is a function of the carrier concentration, one of the determinants of adverse reactions and toxicity (Cabrales et al. 2008).

## 4.3 Plasma Expanders as a Model for HBOC Properties

Plasma expanders have been broadly characterized in terms of: (1) circulating/intravascular time; (2) colloid osmotic pressure (COP) ; (3) ability to reduce blood viscosity; and, (4) lack of RBC aggregation.

Factors that define an optimal plasma expander are prolonged maintenance of circulating volume and central blood pressure, lack of RBC aggregation, effectiveness at low concentration and maintenance of tissue perfusion. How to achieve these properties is a subject of controversy regarding viscosity, COP, and the type of material needed that insures adequate perfusion of all organs. Furthermore what is applicable and necessary for a plasma expander should apply to a HBOC.

Plasma expanders as currently formulated and HBOCs invariably reduce blood viscosity when introduced in the circulation. Although reduction of blood viscosity has been regarded as beneficial since antiquity, Tsai et al. showed that as blood viscosity is reduced by hemodilution, microvascular function is progressively impaired, jeopardizing tissue survival due to microscopic maldistribution of blood flow (Tsai et al. 1998b). Maintenance of microvascular function has been achieved with hypertonic (7.5% sodium chloride solution) small volume resuscitation which reverses endothelial swelling induced by hemorrhagic shock (Mazzoni et al. 1990), improves systemic hemodynamic parameters and partially restores organ perfusion (Cryer et al. 2005; Zakaria el et al. 2006). Clinical trials, however, did not show better outcome using these solutions (Bunn et al. 2004) probably due to concomitant sustained venular constriction (Bouskela et al. 1990). The combination of hypertonic and hyperviscous plasma expander resuscitation provides improved resuscitation in experimental studies when compared to either formulation administered singly (Cabrales et al. 2004b).

An Hb solution formulated to provide $O_2$ carrying capacity, i.e., a HBOC, can be modeled by a colloidal plasma expander of the same physical properties. This approach is useful for identifying the effects and efficacy of a HBOC, independently of the effects due to molecular Hb in the circulation such as nitric oxide (NO) scavenging and the release of heme.

## 4.4 Optimal Plasma Expansion Properties

Maintenance of tissue perfusion is a critical microvascular function, and therefore should be the target for plasma expansion. Functional perfusion is characterized by functional capillary density (FCD) which quantifies capillary perfusion by measuring the total length of capillaries with transit of RBCs during 30 s per unit area of tissue analyzed. This parameter was not evaluated clinically until recently (De Backer et al. 2007; Vincent et al. 2005). Capillary perfusion is usually linked to $O_2$ delivery to the tissue leading to the assumption that maintenance of adequate tissue $PO_2$ reflects normal tissue function, however studies in hemorrhagic shock show that survival is determined by maintenance of FCD and independent of tissue $PO_2$ level (Kerger et al. 1996).

Current plasma expansion formulations do not address their effects on microvascular function. This is partially due to the lack of a mechanistic understanding on the consequences of changing blood composition and properties following their introduction in the circulation on microvascular physiology.

Blood transfusions are successfully used for resuscitation; however, it is not certain that their main contribution during the initial intervention is the restoration of $O_2$ transport capacity. Resuscitation from hemorrhagic shock in awake hamsters with fresh blood, non-oxygen carrying fresh blood equilibrated with carbon monoxide, and blood whose Hb was converted to methemoglobin was equally successful (Cabrales et al. 2007a). Therefore in this process blood contributes to resuscitation by restoring properties unrelated to $O_2$ carrying capacity such as increasing blood viscosity, reduced by decreased hematocrit (Hct), autotransfusion and blood volume restoration by plasma expanders. Hct is the major contributor to blood viscosity, therefore is likely that the transfusion trigger in some instances is actually a "blood viscosity trigger", indicating that there is a portion of anemia that may be treated solely by increasing blood viscosity, an effect that can be achieved by increasing plasma viscosity with a suitable plasma expander. Furthermore the Hct and therefore blood viscosity threshold that causes the decrease of FCD tends to coincide with the decision of transfusing blood.

In terms of the actual effects of a blood transfusions, it is likely that restoring blood viscosity (Cabrales et al. 2005a, 2007a, 2007b) is as important as the provision of additional $O_2$ carrying capacity. Therefore the effect of using RBCs to increase blood viscosity can also be achieved using a viscogenic plasma expander. Also, RBCs increase blood viscosity most effectively in the central circulation, since Hct is significantly reduced from the systemic to the microcirculation. Conversely viscogenic plasma expanders increase blood viscosity throughout the systemic and microcirculation.

## 4.5 Blood Rheology and Blood Flow Regulation

Blood rheology is primarily determined by the exponential relationship between blood viscosity and Hct. In general viscosity is a function of the rate at which fluid is deformed or shear rate. The rate of deformation is a direct function of the applied shear stress, and viscosity is the ratio between shear rate and shear stress. This ratio is constant for a Newtonian fluid like blood plasma, and depends on shear rate for blood, which becomes less viscous with increasing shear rate. This property labeled "shear thinning" causes the velocity profile of blood flowing in a blood vessel to become blunt when compared to the velocity distribution of a Newtonian fluid which is parabolic.

The $O_2$ carrying and delivery capacity of blood are intertwined since carrying capacity is determined by Hct, which influences blood viscosity and therefore blood flow hindrance. In the healthy organism this relationship is auto regulated by mechanotransduction, whereby increased $O_2$ carrying capacity, i.e., Hct and therefore blood viscosity, increases vessel wall shear rate (WSS) and the production of vasodilators by the endothelium (Frangos et al. 1996).

In the circulation, Hct is distributed varying from the systemic value of about 45% in men and 40% in women to about 10% in the capillary circulation as shown in Fig. 4.1. As a consequence the viscosity of blood in the capillary circulation is approximately that of plasma, while systemic blood viscosity is about 4.5 cP (Lipowsky and Firrell 1986). The extension of the capillary circulation and its minimal Hct and $O_2$ partial pressure suggest that tissue oxygenation may not a critical capillary function, with important implications for how to specify the $O_2$ affinity of HBOCs so that they distribute $O_2$ to the microvascular network providing optimal tissue oxygenation.

Blood viscosity in the circulation is a factor in setting peripheral vascular resistance and therefore cardiac output (CO). However, it is not the only fluid related determinant of blood flow hindrance, which also results from the friction caused by flow on the vessel wall, or vessel wall shear stress (WSS) determined in part by the shear thinning properties of the fluid. Shear thinning is caused by aggregation in the fluid components which are dispersed or reduced as shear rate increases. This effect blunts the velocity profile and transfers shear stress related viscous losses from the bulk of the fluid, and particularly the center of tube fluid flow where shear rates are small, to the periphery where they are maximal (Sriram et al. 2012), increasing WSS by comparison to a Newtonian fluid as shown diagrammatically in Fig. 4.2.

Blood viscosity and shear thinning are synergistic since they determine peripheral vascular resistance, CO and therefore blood flow. Blood flow in combination with the viscosity at the vessel wall determines WSS, which modulates the production of vasodilators such as nitric oxide (NO) and prostaglandins (Frangos et al. 1985, 1996). This effect influences the anatomical (geometrical) component of vascular resistance independently of centrally mediated controls.

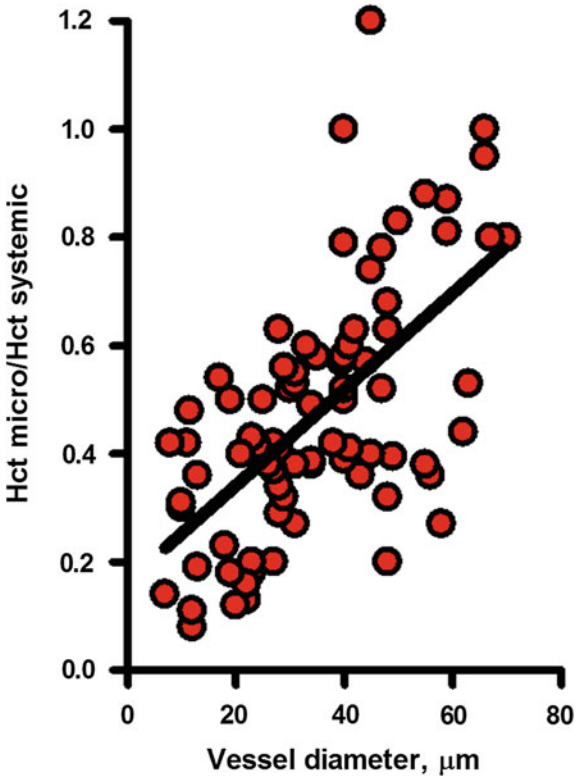


**Fig. 4.1** Distribution of Hct in the microcirculation. Experimental findings in the cat mesentery. The decrease in Hct from arterioles to capillaries causes that the increase in plasma viscosity primarily affects the capillary circulation rather than the larger arterioles and the central circulation data from (Lipowsky and Firrell 1986)

## 4.6 Blood Rheology and Plasma Expansion

Introduction of a plasma expander or a low viscosity HBOC in the circulation causes hemodilution and increases CO. Most plasma expanders in use are mostly Newtonian fluids that have plasma like viscosity (about 1.2 cP). Furthermore they tend to decrease blood's non-Newtonian behavior by lowering aggregation and therefore reducing shear thinning behavior.

The distribution of Hct in the circulation determines that hemodilution with a conventional, low viscosity (about 1.2 cP) plasma expander lowers the viscosity of the central blood vessels, having a significantly lesser effect in the microcirculation, and in practice has no effect in the capillaries. Conversely, moderate hemodilution with a high viscosity plasma expander has proportionally a maximal effect in the large surface area capillary system and a lesser effect in the central blood vessels.

Continuing hemodilution causes the progressively decrease of WSS since viscosity drops more rapidly than the increase in blood flow. Introduction of a HBOC (or a plasma expander) with plasma-like viscosity when Hct is reduced to approximately half, which corresponds approximately to the conventional transfusion

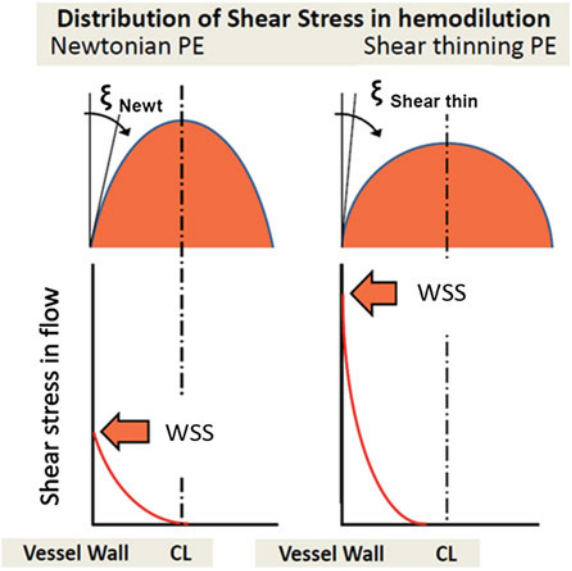


**Fig. 4.2** Hemodilution with a Newtonian and a shear thinning inducing plasma expander (PE). In shear thinning blood core flow has low shear rates and therefore high viscosity, blunting velocity profiles. This increases WSS due to decreased angle $\xi$, inversely proportional to shear rate. In this example shear rate in the shear thinning flow is 2.8 times that in the Newtonian flow. Multiplying shear rate along the velocity profile times viscosity measured at that shear rate yields WSS. Vessel diameter vasodilation effect of +20% due to increased NO production is also shown, counteracting the increase of shear rate. Maximal flow velocity decreases but blood flow increase due to the change in velocity profile and vasodilation

trigger, will further decrease blood viscosity and WSS causing vasoconstriction due to reduced mechanotransduction.

Vasoconstriction has a collateral effect since it curtails transmission of central blood pressure to the capillaries, lowering capillary pressure, causing their collapse and decreasing FCD, effects that have been documented by direct in vivo studies of the microcirculation.

A key aspect of the use of a low viscosity HBOC is that independently of its $O_2$ carrying properties, its introduction at the transfusion trigger is identical to continuing the hemodilution process. Thus there is a vasoconstriction response in the circulation that is solely due the lowering of WSS which reduces vasodilator production, independently of the innate property of molecular Hb to scavenge NO.

## 4.7 Counteracting Vasoconstriction with Supra-Perfusion

Increasing plasma viscosity in hemodilution is a proven procedure for increasing WSS. This effect is attained with plasma expanders that increase plasma viscosity to about 2.5–2.8 cP which even with Hct at about half of normal, causes overall

blood viscosity to be lower than normal which leads to a significant increase in CO. This increase in CO in combination with the higher viscosity significantly increases WSS. In the healthy organism, with normal endothelial function the increase of WSS increases the production of NO, particularly in the microcirculation, the area with the largest endothelia surface in the circulation.

The significant increase in NO (Sriram et al. 2012; Tsai et al. 2005) causes vasodilatation, which in combination with lowered over all blood viscosity results in condition of supra-perfusion characterized by comparatively high FCD and capillary flow velocities that are 25–50% above baseline. These effects become particularly evident when systemic Hct is reduced 75% of baseline normal levels and cannot be obtained unless plasma viscosity and/or WSS are increased.

Supra-perfusion from increased plasma viscosity hemodilution re-distributes pressure in the circulation (Mirhashemi et al. 1987b), increasing pressure in the capillaries which is critical for maintaining FCD as shown by direct measurements of capillary pressure (Cabrales et al. 2004c). An important additional systemic effect due to lowered Hct and overall blood viscosity occurs in the venous return where central venous pressure is increased, improving cardiac performance and increasing CO (Messmer et al. 1972). It should be noted that this synergy between viscogenic plasma expansion, pressure redistribution and increased mechano-transduction is not common to all viscogenic plasma expanders (Cabrales et al. 2005b), appearing to be a feature of colloidal plasma expanders based on relatively large molecules (Sakai et al. 2000).

## 4.8 Blood Viscosity, Hemodilution and Viscogenic Plasma Expansion

Studies of blood rheology tend to demonstrate that increased blood and/or plasma viscosity result from or lead to pathological conditions. However, there is growing evidence supporting that increased plasma viscosity is not detrimental and may be beneficial. Chen et al. (Chen et al. 1989) elevated plasma viscosity fourfold (4 cP) finding vasodilation and reduction of vascular hindrance in several vital organs. Waschke et al. (Waschke et al. 1994), found unchanged cerebral perfusion when blood was replaced with fluids with the same $O_2$ carrying capacity and viscosities varying from 1.4 to 7.7 cP. Krieter et al. (1995) infused dextran 500 kDa and found maximal tissue $PO_2$ in skeletal muscle and liver at plasma viscosities of 3 and 2 cP respectively. De Wit et al. (1997) found that elevation of plasma viscosity elicited sustained NO-mediated dilatation in the hamster muscle microcirculation.

Substitution of RBCs with a colloidal or crystalloid solution is safe up to exchanges of 50% of the RBC mass (Tuma 1989). A 50% decrease in Hct brings the Hb concentration to the transfusion trigger, usually about 7 g Hb/dl. At this Hct, tissue $O_2$, blood pressure and FCD are normal. Microvascular conditions change when this threshold is passed (Intaglietta 1989), leading to lowered FCD.

Thus, maintain rheologic properties of blood during resuscitation from hemorrhagic shock is a factor in restoring organ perfusion. Wang et al. could not maintain CO using 2x, 3x and 4x shed blood volume of Ringer's lactate in hemorrhagic shock resuscitation in rats, although total peripheral resistance was restored with the 4x infusion (Wang and Chaudry 1991). However Ringer's lactate 4x infusion did not restore or maintain microvascular flow in the liver, spleen, skeletal muscle and small intestine (Wang et al. 1990).

It is important to consider that attainment of the beneficial effect of viscogenic plasma expansion requires a normal vascular endothelium with functional glycocalyx that responds to mechanical stimuli. This condition may not be present in clinical situations involving older subjects (Herrera et al. 2010), especially those with underlying cardiovascular pathologies such as diabetes and atherosclerosis, associated with altered glycocalyx and endothelial dysfunction. High viscosity plasma expanders increased the heart's workload since it has to pump a higher viscosity blood. This may also limit its application in higher risk patients afflicted with cardiac failure/dysfunction. Therefore this approach may be more directly applicable in military medicine, in the treatment of military battlefield casualties, a population that is generally young and physically fit.

## 4.9 The Viscosity of Colloidal Plasma Expanders

Historically non-crystalloid plasma expanders were formulated to limit their viscosities. Gelatin, dextran and starch solutions have viscosities in the range of 2 cP prior to administration. The inherent dilution of materials upon introduction into the circulation determined that final plasma viscosity was similar to normal, i.e., about 1.0–1.2 cP. Hemoglobin based oxygen carriers (HBOC) were also formulated to limit their viscosity.

The viscosity of a colloidal solution is determined by the number of particles per unit volume and the solute molecular volume. Therefore augmenting concentration of low molecular volume species to increase viscosity of plasma expanders increases COP, bringing interstitial fluid into the circulation, diluting the material, lowering viscosity, a self-limiting process.

Viscogenic plasma expanders are: (1) *Hydroxyethylstarch* (HES; Voluven®, 6%, 140 kDa), Pentaspan®, 10%, 200 kDa), Hextend®, 6%, 500 kDa). The viscogenic effects of these materials have a relatively short duration due to their high COP. (2) *Polyvinylpyrrolidone* (PVP; ~1.1 MDa) causes immune and inflammatory reactions related to the distribution of molecular weights in the compound when studied in the brain circulation (Rebel et al. 2001) and is not completely eliminated from the organism. It is no longer approved for human use in the US. (3) *Dextran,* namely branched polysaccharides (40, 70, 500 kDa). They present a small probability of causing anaphylactic shock (Michelson 1968) and Dextran >250 kDa causes RBC aggregation when blood Hct is near normal. (4) *Alginate*. A polysaccharide derived from seaweed with high viscosity at low concentrations

(0.7% solution has viscosity of 8 cP and virtually no COP) used extensively in biotechnology due to its water-binding and viscosifying properties. They present a mixture of M (manuronic acid) and G (glucuronic acid) and their applicability depends on optimizing the relative concentrations (Cabrales et al. 2005c; Ertesvåg et al. 1999). (5) *Keratin*. Alpha-keratin derived from human hair was used successfully in exchange transfusions in dogs (Ewald et al. 1964). (6) *Pegylated proteins*, a class of large volume molecules generated by attaching polyethylene glycol (PEG) chains to the surface amino groups of proteins (Cabrales et al. 2005e).

Table 4.1 summarizes the viscosity and COP of several plasma expanders that are currently used clinically or are being investigated as potential next generation fluids. Additionally summarized in this table are the results from microvascular studies when the fluids were used to extend the transfusion trigger in a hemodilution protocol. Functional capillary density achieved during this state correlates with the plasma viscosity after the study fluid was exchanged and Hct lowered to 11%, an "extreme hemodilution" state. A closer inspection of the results further suggests that a viscosity threshold needs to be established in order to attain the higher FCD levels.

Each one of these molecular species can serve as a design model for proposed modifications of Hb to be used in HBOCs. Albumin is a particularly suitable globular protein that can be polymerized and conjugated with PEG to reproduce the physical effects of polymerizing Hb and conjugating Hb with PEG independently of the $O_2$ transport properties (Cabrales et al. 2005d).

**Table 4.1** Plasma expander properties. After extreme hemodilution: plasmaproperties and functional capillary density

| | Solution Viscosity (cp) | Solution COP (mmHg) | Plasma Viscosity (cp) | FCD (relative to baseline) |
|---|---|---|---|---|
| HSA, 5 %[a] | 0.9 | 21 | 1.17 | 0.49 |
| Mal-Propyl-P5K6-HSA, 4 %[b] | 2.2 | 42 | 1.8 | 0.67 |
| Dextran 70, 6 %[c] | 2.8 | 50 | 1.4 | 0.38 |
| HES 200/0.6, 10 %[d] | 4.3 | 99 | 1.3 | 0.66 |
| Dextran 500, 6 %[c] | 5.9 | 32 | 2.2 | 0.71 |
| Alginate, 0.7 %[e] | 8.8 | 31 | 2.7 | 0.76 |

[a] Human Serum Albumin (Baxter). Sakai et al., 2005, Am J Physiol Heart Circ Physiol.
[b] PEGylated Human Serum Albumin (Sangart). Cabrales et al., 2005c, d, eAm J Physiol Heart Circ Physiol.
[c] Tsai et al., 1998a, b, Am J Physiol Heart Circ Physiol.
[d] Hydroxyethyl starch, Pentaspan (B. Braun). Cabrales et al., 2005a, b, c, d, eAnesthesia.
[e] Alginate (Novamatrix/FMC Biopoymer) , with Dextran 70, 4% to adjust COP. Cabrales et al, 2005c, d, eAm J Physiol Heart Circ Physiol

## 4.10 PEG-Albumin, a Low Viscosity Plasma Expander that Causes Supra-Perfusion

PEGylated proteins are a family of molecules that produce supra-perfusion when used as plasma expanders, although they are not particularly viscogenic. PEG-Albumin (PEG-Alb, 4 % protein concentration with viscosity of 2.2 cP) used for extreme hemodilution yields a FCD of 62% despite only elevating plasma viscosity to 1.3 cP (Tsai et al. 2007) as shown in Fig. 4.3. Notably CO increased 50% above baseline (Cabrales et al. 2005e) establishing conditions of supra-perfusion. Although PEG-Alb does not increase blood bulk viscosity, it does increase WSS and the production of NO by the endothelium. This result is a consequence of PEG-Alb restoring the shear thinning properties of blood, with the additional advantage of maintaining a low bulk blood viscosity, thus facilitating cardiac function (Sriram et al. 2012). The effect by which shear thinning in blunting the velocity profile increasing WSS is augmented by the cell free layer whose low viscosity (relative to that of core flow blood) further increases shear rate and shear stress at the wall.

Studies with PEG-Hb show similar effects, even though this molecule scavenges NO resulting in a low NO concentration in the vascular wall when used in identical hemodilution conditions as PEG-Alb, which shows 2–3 fold increase in microvascular wall NO concentration (Tsai et al. 2006). This anomalous effect is probably not related to additional NO originating from nitrite reductase activity (Lui et al. 2008; Lui and Kluger 2009) or ATP (Ellsworth et al. 2009) released from RBCs since direct measurements of vessel wall NO concentration show that this is low (Tsai et al. 2005) due to the NO scavenging properties of Hb.

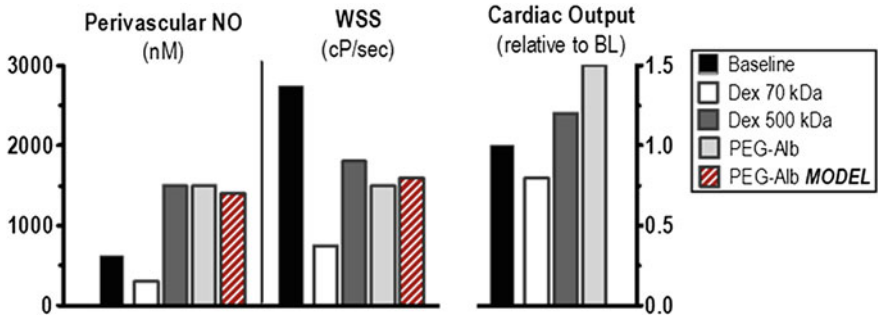


**Fig. 4.3** PEG-Alb, a low viscosity plasma expander investigated experimentally in extreme hemodilution (Hct 11%) and analytical modeling § (Sriram et al. 2012). Results are compared with data from Dextran 70 kDa and 500 kDa, low and high viscosity plasma expanders (Tsai et al. 2005; Tsai et al. 1998b). Baseline: whole blood. PEG-Alb leads to similar WSS and vessel wall NO bioavailability as the high viscosity plasma expander, even though the resulting plasma viscosity is significantly lower (1.3 vs. 2.2 cp). This effect is due to shear thinning of the blood/PEG-Alb mixture. NO bioavailability is greater than in blood because increased cell free layer dimension limits NO scavenging by Hb. Dex 70 kDa, due low viscosity does not cause sufficient WSS

It should be noted that NO is not the only vasoactive mediator released from the endothelium by shear stress. The original experiments on mechanotransduction by Frangos et al. (Frangos et al. 1985) and Grabowski et al. (1985) showed that shear stress promoted the release of prostaglandins, and Koller and Kaley (1990) showed that this effect had a strong vasodilator effect. This phenomenology that is independent of the management of NO has not been explored in relation with the development of HBOCs which primarily focused on the NO management by the presence of Hb in plasma and low viscosity solutions and therefore low WSS conditions.

## 4.11 Plasma Expansion and $O_2$ Transport and Availability

Hemodilution decreases blood $O_2$ carrying capacity which viscogenic plasma expansion compensates for by significantly increasing flow velocity. $O_2$ is delivered by convection through and diffusion out of the blood vessels. Increasing flow velocity increases $O_2$ convection and allows for an increased capillary $O_2$ since $O_2$ diffusion or exit rate remains constant.

Vessel wall NO concentration increases significantly with PEG-Alb hemodilution due to increased endothelial production and decreased scavenging due to lower Hb blood concentration and an increased cell free plasma layer width, distance between the endothelial cell source and the RBC column sink. Increased NO bioavailability also affects $O_2$ metabolism as shown in conscious canines at rest and during exercise, where inhibition of NO synthesis increased $O_2$ consumption at all levels of external work, independent of the change in skeletal muscle blood flow (Shen et al. 2000). Human skeletal muscle blood flow and $O_2$ uptake measured using positron emission tomography show that inhibition of NO production enhances resting muscle $O_2$ uptake by 20%.

However cell respiration is inhibited when inducible NO synthase increases NO generation (Bolaños et al. 1997). Decreasing $O_2$ consumption through stimulation of NO synthesis is how angiotensin-converting enzyme inhibitors (enalapril) increase intrarenal $O_2$ tension (Adler and Huang 2002). Dietary nitrate supplementation produces NO synthase-independent NO (Gladwin et al. 2005) increasing circulating nitrite (Lundberg and Govoni 2004) forming NO, reducing $O_2$ consumption during maximal arm and leg exercise (Larsen et al. 2010). Therefore studies evidence that increased NO concentration lowers $O_2$ metabolism and vice versa. The significance of the NO–$O_2$ metabolism relationship is that increased NO bioavailability may reduce $O_2$ demand which is equivalent to increasing $O_2$ carrying and delivery capacity.

## 4.12 Effect of Viscogenic Plasma Expansion on Cardiac Function

The heart plays a central role in plasma expansion since it has to be able to respond to the decreased peripheral vascular resistance by increasing CO. Increasing plasma viscosity affects heart function differently depending on whether we consider the heart as a tissue or an organ. Heart function is presumably improved due to viscous plasma perfusion since like all other tissues it should show vasodilation and therefore increased perfusion of the heart muscle. Conversely, viewed as an organ, increasing plasma viscosity increases the workload and energy requirements, as more fluid with greater viscosity is pumped at a higher rate.

Increased plasma viscosity improves muscle tissue perfusion and therefore heart function, however the increased blood viscosity will increase cardiac workload by comparison to lower viscosity blood when delivering the same blood flow to the tissues due to increased vascular resistance. For healthy young subjects, the heart has sufficient functional reserve capacity so that it will increase its contractility to meet the increased workload. However, the heart of subjects with significant underlying cardiovascular disease, has limited functional reserve to deal with the increased workload (Cotter et al. 2002) therefore this approach requires a careful assessment of benefit-risk before use in human subjects.

It should be noted that the increase in plasma viscosity with a viscogenic plasma expander is inevitably associated with the decrease of Hct, therefore viscogenic hemodilution and the decrease of blood viscosity, are compensating effects that in practice tend to keep overall blood viscosity constant. Since the objective of increasing plasma viscosity is that of increasing $O_2$ delivery capacity through the induction of supra perfusion, a comparison between the available approaches and related risks should include alternatives, namely HBOCs and blood. As previously discussed, HBOCs must contend with the effects of low viscosity, WSS and NO leading to vasoconstriction, while a blood transfusion (packed RBCs) will significantly increase blood viscosity. Therefore increasing plasma viscosity to twice its normal value, which is sufficient to elicit the supra-perfusion effect, in general will not result in an increase of blood viscosity beyond the normal value.

Cardiac function studies in small animal were made using a miniaturized conductance catheter for real-time measurements of pressure and volume in the left ventricle. These experiments allowed evaluating left ventricular function, independent of load conditions and heart rate (Georgakopoulos et al. 1998; Nishio et al. 2002; Pacher et al. 2004; Westermann et al. 2008). Comparing the effects of low and high viscosity plasma expanders on heart function, these studies showed that PEG-Alb causes a significant improvement on cardiac performance during hemodilution and resuscitation from hemorrhagic shock compared to conventional and high viscosity colloids.

The cardiac effects of PEG-Alb are due to decreased systemic vascular resistance, and increased on CO and stroke volume (SV) because of increased heart

energy efficiency in ejecting blood diluted with PEG-Alb compared to plasma expanders with higher viscosities, as indicated by the decreased work needed to eject a unit of volume. In hemodilution and shock resuscitation studies, CO was increased with PEG-Alb (Cabrales et al. 2005b, 2005e; Martini et al. 2008; Winslow et al. 2004). The increase in CO after hemodilution and resuscitation from hemorrhagic shock with PEG-Alb is due to decreased blood viscosity and compensatory mechanism to reduced blood $O_2$ carrying capacity to maintain adequate tissue oxygenation and organ function (Mirhashemi et al. 1987a).

Studies of hemodilution and resuscitation from hemorrhagic shock demonstrated that PEG-Alb increased stroke work as compared to other plasma expanders, indicating that PEG-Alb provides sufficient $O_2$ to permit the heart to increase its function. Conversely, infusion of high viscosity plasma expanders gradually decreased stroke work over time, indicating a lack of sufficient energy to maintain ejection of blood. Microvascular studies during hemodilution with PEG-Alb have shown an increase in $O_2$ delivery and extraction when compared to other plasma expanders (Cabrales et al. 2005d). A similar response can take place at the heart microvasculature, explaining the superior mechanoenergetic responses produced by PEG-Alb compared to higher viscosity plasma expanders.

Ejection of blood diluted with PEG-Alb presented a lesser functional load to heart, lowering the energy consumed per unit volume by comparison with viscous plasma expanders. Moreover, during resuscitation from hemorrhagic shock, PEG-Alb also sustained a higher energy to blood ejected per unit of volume compared to resuscitation with plasma expanders with higher viscosity. Counter intuitively, PEG-Alb resuscitation from hemorrhagic shock improved and maintained load-dependent parameters (e.g. $dp/dt_{max}$, $dp/dt_{min}$ and $dv/dt_{max}$) compared to viscous plasma expanders. This result implies an increase in contractile function and volumetric change, and that low viscosity active plasma expanders yield significant beneficial effects during resuscitation (Chatpun and Cabrales 2010a, 2010b).

## 4.13 Summary and Conclusions

Plasma expanders are generally considered a mature, well established and clinically well integrated technology, which may be the case if their sole purpose is the restoration and maintenance of circulating volume. In this scenario plasma expansion is a process to be followed by blood transfusion once it reaches a limit usually perceived to be due to the deficit on $O_2$ carrying capacity, but that in many cases may reflect the failure of microvascular function. It should be apparent that substituting blood with a HBOC in the resuscitation sequence that starts with plasma expansion to maintain blood volume will not be fully effective since in general HBOCs lack the fluid mechanical properties with which blood causes the microcirculation to be functional. In fact it is likely that many HBOC formulations result in fluid mechanical properties that are the same as those of conventional plasma expanders that cannot be effectively used beyond the transfusion trigger.

It is apparent that in formulating HBOCs concentration of $O_2$ carrier the resulting viscosity of the circulating blood, COP and blood shear thinning must be specifically related. In this context it becomes important to define the transfusion process in which the HBOC will be used, i.e., whether it will be administered from the beginning of the blood volume restoration intervention. Alternatively treatment may be started with a plasma expander and continued with HBOCs when the perceived safe limit of $O_2$ carrying capacity deficit is reached. In either case a condition of microvascular collapse will be reached regardless of the circulating $O_2$ carrying capacity unless blood viscosity and WSS are restored to near normal values or preferentially enhanced in the microcirculation.

It should be noted that in reality plasma expanders that are able to sustain microvascular function in the presence of significant blood losses are not yet available, although sufficient data exists from experimental studies to specify and formulate their characteristics.

Experimental results suggest that an optimal plasma expander should have shear thinning properties *per se*, as well as inducing shear thinning in diluted blood. This combination of properties is probably more desirable than just using a Newtonian high viscosity fluid, since it would tend to lower the heart workload. The actual viscosity of these plasma expanders should be formulated so that the mixture of plasma expander and blood never exceeds the viscosity of normal blood.

These plasma expanders should induce the increase of CFL dimensions, since this results in higher WSS. The COP of this plasma expander should be near normal, considering that recipients may present some degree of hemodilution due to auto-transfusion and consequent lowered circulating plasma COP. Adding a fluid with near normal COP minimizes fluid shifts and allows the prediction of the evolution of rheological effects in the patient's circulation with greater accuracy.

Availability of this type of plasma expander should be of significant benefit in transfusion medicine because it potentially extends the margin of safety afforded by plasma expansion in postponing blood and HBOC transfusions. Furthermore it would serve as a model for the fluid mechanical properties necessary for designing HBOCs, which should be formulated as $O_2$ carriers with optimal plasma expansion properties, since otherwise poor plasma expansion negates the benefits of additional $O_2$ carrying capacity. Future developments for extending the applicability these fluids will benefit from the development of indicators for obtaining a deeper understanding of the cardiovascular functional status of the individual in treatment an approach that parallels that discussed in Chap. 6.

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# Part II
# Hemorrhagic Shock and Current Treatments

# Chapter 5
# Pathophysiology of Hemorrhagic Shock and Resuscitation

**Fredric M. Pieracci and Walter L. Biffl**

Shock may be defined broadly as a condition in which metabolic energy production is limited either by the supply or utilization of oxygen, manifest by derangement of the normal oxygen supply–demand balance, accumulation of the byproducts of anaerobic metabolism, and dysfunction of one or more organ system. In the case of massive hemorrhage, shock is due specifically to impaired oxygen delivery ($VO_2$) secondary to both hypovolemia and anemia. Restoration of tissue perfusion, termed resuscitation, proceeds systematically, is based upon the underlying etiology of shock, terminates upon achievement of clearly defined endpoints, and requires frequent re-evaluation. Resuscitation has been refined substantially over the previous decade, with a resultant improvement in the outcomes of critically ill patients (Brun-Buisson et al. 2004; Martin et al. 2003). Major changes have included improved accuracy of the assessment of intravascular volume status, recognition of the detrimental effects of both allogeneic blood product transfusion and excessive volume expansion, and timely, goal-directed treatment of shock and its complications. This chapter will focus on the diagnosis of shock, differentiation into hemorrhagic shock, the benefits and limitations of various measurements used to determine the adequacy of resuscitation, general resuscitative strategies, and complications of resuscitation.

## 5.1 Pathophysiology and Etiologies of Shock

Throughout medical history, the notion of shock has been captured using many colorful terms, ranging from "a momentary pause in the act of death" (Warren 1952) to "the rude unhinging of the machinery of life" (Gross 1872). At the

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cellular level, shock represents inadequate tissue perfusion resulting in compromise of one or more organ systems. Oxygen delivery that is insufficient to meet metabolic demands is believed to be the main defect responsible for shock, although impaired oxygen utilization despite normal or even supranormal $VO_2$ has been observed in septic shock specifically. Although hypotension is an important component of shock, substantial tissue hypoperfusion may exist despite a "normal" ($\geq$60 mm Hg) mean arterial pressure (Wo et al. 1993), so-called *occult hypoperfusion*, underscoring the importance of multiple measurements of tissue perfusion.

Oxygen delivery is the product of arterial oxygen content and cardiac output. Cardiac output, in turn, is dependent upon intravascular volume, cardiac contractility, and systemic vascular tone. Derangement of these parameters results in three broad categories of shock: hypovolemic, cardiogenic, and vasodilatory, respectively, although less common etiologies of shock are recognized. Hemorrhagic shock is considered traditionally as a sub-classification of hypovolemic shock, as the etiology of hypovolemia is due specifically to blood loss. However, impaired tissue perfusion in hemorrhagic shock is actually due to both hypovolemia (decreased preload) and anemia (decreased arterial oxygen content). This distinction has obvious therapeutic implications in that both volume and oxygen carrying capacity must be restored.

Circulating blood volume (L) is approximately 7 % of body weight (kg): A 70 kg individual thus possesses a blood volume of approximately 4.9 L. A classification schema espoused by the American College of Surgeon's Advanced Trauma Life Support recognizes four classes of hemorrhage based upon absolute quantities of blood loss (Table 5.1). Although this classification schema is useful for understanding the serial effects of increasing hemorrhage on physiology, it does not account for the marked inter-patient variability in the degree of hemorrhage that is necessary to cause shock. A myriad of co variables, including age, comorbid conditions (particularly cardiopulmonary disease), and baseline hemoglobin concentration, influence this quantity. Thus, a more accurate definition of hemorrhagic shock involves any quantity of acute blood loss that results in evidence of end organ hypoperfusion.

**Table 5.1** Advanced trauma life support classification of hemorrhage and resultant physiologic derangements

| Class | Blood Loss (mL) | Pulse rate | Blood pressure | Pulse pressure | RR | Mental status |
|---|---|---|---|---|---|---|
| I | Up to 750 | Normal | Normal | Normal | Normal | Slightly anxious |
| II | 750–1,500 | 100–120 | Normal | Decreased | 20–30 | Anxious |
| III | 1,500–2,000 | 120–140 | Decreased | Decreased | 30–40 | Confused |
| IV | >2,000 | >140 | Decreased | Decreased | >40 | Lethargic |

Numeric values are for a 70 kg male. RR, respiratory rate

## 5.2 Markers of Tissue Perfusion and Endpoints of Resuscitation

In order to minimize the complications of overzealous volume expansion, shock must be diagnosed prior to embarking on resuscitation. Although the diagnosis of shock is often based upon an overall impression by an experienced clinician, it is helpful to document the presence of shock in terms of objective measurements of tissue perfusion. Concern for shock is often raised initially in the face of organ system dysfunction. Major organ systems and associated signs of hypoperfusion are listed in Table 5.2. The main limitation of measurements of individual organ function is that derangement may be due to intrinsic disease as opposed to hypoperfusion, mandating measurements of global tissue perfusion.

Multiple markers of global tissue perfusion exist; they may be grouped broadly into measurements of either the amount of oxygen delivered to the tissue (*upstream markers*) or the adequacy of tissue $DO_2$ given the level of metabolic demand (*downstream markers*). Examples of upstream markers include measurements of intra-vascular volume status, cardiac output, and direct calculation of $DO_2$. Examples of downstream markers include measurements of either oxygen extraction or anaerobic metabolism. Whereas upstream markers are useful for gauging which aspect of circulatory system derangement requires therapy (*i.e.*, intravascular volume, cardiac contractility, or vascular tone), downstream markers generally identify a global problem with perfusion, as well as inform the response to resuscitative interventions (*i.e.*, the adequacy of resuscitation).

Measurements of volume status may be dichotomized as either static or dynamic. Static measurements record a point estimate of absolute intravascular volume status which is then extrapolated to assess preload responsiveness; whereas lower values are indicative of hypovolemia, higher values imply volume overload. In general, intravascular pressure (either venous or pulmonary arterial) is used to approximate volume. Central venous pressure (CVP) and pulmonary artery occlusion pressure (PAOP) are the most commonly used static measurements of intravascular volume status.

**Table 5.2** Signs and symptoms of organ hypoperfusion

| Organ system | Sign/symptom |
|---|---|
| Central nervous | Lethargy, agitation, coma |
| Pulmonary | Acute respiratory distress syndrome |
| Cardiovascular | Hypotension, ST segment depression/elevation, elevation of cardiac enzymes |
| Renal | Oliguria, decreased fractional excretion of sodium |
| Gastrointestinal | Feeding intolerance, adynamic ileus, stress ulceration/gastritis, enteritis, colitis |
| Hepatopancreaticobiliary | Hepatitis, centrilobar necrosis, cholestasis, acalculous cholecystitis, pancreatitis |

The principle limitation of static measurements is that both inter and intra-patient variability of Frank-Starling curves renders interpretation of absolute measurements inaccurate. For example, a patient with an elevated CVP may still be operating on the steep portion of the Frank-Starling curve. Conversely, a patient with a low CVP need not remain preload responsive. Beyond this limitation, static measurements of volume responsiveness are influenced by several additional factors, including changes in intra-thoracic pressure, valvular disease, and pulmonary hypertension.

In contrast to static measurements, dynamic measurements of volume responsiveness exploit physiologic variations in pre-load in an attempt to determine the efficacy of volume expansion. A determination of preload responsiveness is thus made irrespective of the underlying absolute volume status. Such "natural" variations in preload made be divided broadly into respiratory variations, such as pulse pressure variation (PPV), systolic pressure variation (SPV), and stroke volume variation (SVV), and positional variations, such as those induced by passive leg raise (PLR). Respiratory variation in arterial pressure involves the increase in preload that occurs during passive mechanical ventilation, followed by a decrease in preload during expiration. These differences in preload translate into differences in cardiac output, whether measured as SPV, PPV, or SVV. Variability in preload, and hence cardiac output, is more pronounced when the heart operates on the steep portion of the Frank-Starling curve, such that an increased variability corresponds to preload responsiveness. The transfer of effective blood volume from the capacitance bed of the lower extremity induced by PLR results in a similar increase in filling pressure, the effect of which is again dependent upon preload responsiveness. Although dynamic measurements of intra-vascular volume are not without their limitations, their superiority of static measurements has been demonstrated convincingly (Michard and Teboul 2002; Marik et al. 2009), and they are preferred whenever possible.

Intravascular volume status represents only one parameter involved in determining tissue perfusion. Measurement of cardiac output is useful when evidence of shock persists despite optimization of volume status. Although cardiac output has been measured traditionally via thermodilution, both echocardiography (Maslow et al. 1996) and pulse contour analysis (de Vaal et al. 2005; McGee et al. 2007) have emerged as non-invasive alternatives with acceptable performance characteristics. Knowledge of the cardiac output, mean arterial pressure, and CVP also allows calculation of the systemic vascular resistance, a parameter that is useful primarily for determining the etiology of shock as opposed to guiding resuscitation.

The most specific upstream measurement of tissue perfusion is $DO_2$. Oxygen delivery is dependent upon cardiac output, arterial hemoglobin concentration and saturation, as well as the partial pressure of oxygen in arterial blood. Importantly, whereas transfusion of allogeneic RBCs likely increases $DO_2$, its effect on $VO_2$ is less clear, with the majority of studies reporting either unchanged or decreased $VO_2$ following transfusion (Kiraly et al. 2009; Tinmouth et al. 2006; Napolitano et al. 2009). Although low $DO_2$ suggests tissue hypoperfusion, the calculation

suffers from the same aforementioned limitations of static measurements, and resuscitation guided by driving $DO_2$ to normal or even supernormal levels is both impractical and does not necessarily improve outcomes (discussed below).

As compared to upstream markers, downstream markers measure adequacy of tissue perfusion with respect to oxygen supply-demand balance, making them more useful for diagnosing shock, assessing its severity, and tracking response to therapy. In general, downstream markers capture the magnitude of either tissue oxygen extraction or anaerobic metabolism.

During normal metabolism, $VO_2$ far exceeds consumption, such that $DO_2$ is independent of $VO_2$ over a wide range of values. Either decreased $DO_2$ or increased $VO_2$ is compensated for by increased oxygen extraction from arterial blood, resulting in a decreased oxygen saturation of venous hemoglobin as it exits the tissue bed. Low venous hemoglobin oxygen saturation is thus an early marker of tissue hypoperfusion. Venous hemoglobin oxygen saturation is measured readily using catheter-based fiber-optic reflective spectroscopy in either the pulmonary artery ($SvO_2$) or superior vena cava ($ScvO_2$) with acceptable correlation (Ladakis et al. 2001; Reinhart et al. 2004). Both $SvO_2$ and $ScvO_2$ reflect flow-weighted pooling of venous blood from multiple tissue beds with variable metabolic requirements. A normal value thus does not rule out adequate oxygenation of all organ systems. However, a low $SvO_2$ (<65 %) is highly suggestive of tissue dysoxia (Krafft et al. 1993). The $ScvO_2$ was a useful endpoint of resuscitation in a study of protocolized, goal-directed therapy of septic shock by Rivers et al (Rivers et al. 2001).

Once $DO_2$ falls below a critical level, compensation via increased oxygen extraction is no longer sufficient, an oxygen debt develops, and anaerobic metabolism ensures. Below this critical level, $VO_2$ is dependent upon $DO_2$, and an increase in $DO_2$ will result in corresponding increase in $VO_2$.

The three byproducts of mammalian anaerobic metabolism are lactate, pyruvate, and hydrogen ion. The arterial lactate concentration is thus proportional directly to oxygen debt. Both elevation and failure of normalization of the serum lactate concentration are highly predictive of adverse outcomes among critically ill patients in shock (Alonso et al. 1973; Moomey et al. 1999; Suistomaa et al. 2000; Abramson et al. 1993). However, lactate is a non-specific marker of global tissue hypoxia. This is because several other conditions, including muscle hyper activity, seizure, accelerated aerobic gylcolysis, and liver disease, may result in elevation of the lactate concentration, regardless of the presence of shock. Moreover, lactate clearance lags several hours behind improvement in tissue oxygenation, rendering the serum lactate concentration problematic for real-time resuscitative efforts.

Hydrogen ion concentration may be estimated by the serum bicarbonate concentration, arterial pH, or base deficit. The base deficit is defined as the amount of base (millimoles/L) required to titrate whole blood to a normal pH at normal values of temperature, $P_aCO_2$, and $P_aO_2$. Elevations of the base deficit beyond the normal range of $-3$ to 3 correlate with the presence and severity of shock (Davis et al. 1988; Rutherford et al. 1992). The base deficit normalizes rapidly following restoration of tissue perfusion, making it an ideal marker for resuscitation.

Measurement of the serum bicarbonate concentration may be used as surrogate for the base deficit with reasonable correlation (Eachempati et al. 2003; Martin et al. 2005) and does not require an arterial sample. Importantly, all measurements of serum acid are rendered inaccurate in the setting of exogenous bicarbonate administration. Furthermore, changes in minute ventilation must be appreciated as variability in $P_aCO_2$ contributes to the serum acid concentration.

Beyond tissue hypoperfusion, several additional cause of acidosis may co-exist in the critically ill patient, such as alcohol intoxication and hyperchloremia. Specifically, large-volume saline resuscitation results in a non-anion gap, hyperchloremic, metabolic acidosis and hence a persistently elevated base deficit despite normalization of perfusion (Kellum et al. 1998; O'Dell et al. 2007). Partitioning of the base deficit addresses this last issue (Fidkowski and Helstrom 2009).

## 5.3 Resuscitation Strategies

Several resuscitation strategies for hemorrhagic shock specifically are discussed below. However, we begin with general points regarding the conduct of resuscitation, regardless of etiology. Initial management of the patient in shock involves a rapid assessment of the relative contributions of the various aforementioned etiologies. Assessment typically proceeds in the following manner: (1) intravascular volume status (including hemoglobin concentration), (2) cardiac contractility, (3) vascular tone. In reality, multiple diagnostic maneuvers are often undertaken simultaneously. Correction of pathology occurs in a similar fashion, optimizing pre-load, followed by cardiac contractility, and finally vascular tone. This sequence is based upon the fundamental principles of cardiodynamics; efforts to improve either contractility or vascular tone will be ineffective in the face of hypovolemia. Furthermore, vasoactive medications offer little benefit if cardiac output is insufficient to perfuse end organs, and may even impede marginal cardiodynamics by increasing afterload. Resuscitation is a dynamic process that mandates frequent re-evaluation. Additional etiologies of shock frequently compound the initial insult. Resuscitation must also be monitored carefully to avoid the complications of overzealous volume expansion.

### *5.3.1 Timing of Resuscitation*

It is intuitive that timely restoration of tissue perfusion will lead to improved outcomes. Animal models of hemorrhagic shock have demonstrated that tissue damage becomes irreversible beyond a critical period of hypoperfusion, after which time restoration of perfusion is superfluous (Shires et al. 1964). These observations have led to the concept of the *resuscitation window*, beyond which

efforts to normalize perfusion are met with diminishing returns. Numerous data now support the existence of the resuscitation window. Kern et al. conducted a meta-analysis of 21 randomized trials comparing goal-directed therapy to conventional management of patients in shock (Kern and Shoemaker 2002). The majority of studies involved optimization of PAOP, cardiac output, and $DO_2$ to either normal or supranormal levels. A benefit to such therapy was observed only among those studies that maximized oxygen delivery either before or early after the onset of organ dysfunction. Early efforts to optimize tissue perfusion have been shown to be of benefit even in the absence of goal achievement, suggesting that the timing of resuscitative efforts, rather than attainment of an arbitrary goal, contributes to outcome benefit (Lobo et al. 2000).

How long, then, does such a resuscitation window remain open? Gattanoni et al. demonstrated no survival benefit to hemodynamic optimization attempted 12–36 h after the onset of organ failure (Gattinoni et al. 1995). By contrast, Rivers et al. documented significant mortality improvement with protocol-driven resuscitation within 6 h of presentation to the emergency ward (Rivers et al. 2001). Thus, resuscitation should begin as early as possible; hypoperfusion intervals of greater than 6–12 h likely cannot be reversed completely with subsequent hemodynamic optimization.

### *5.3.2 Permissive Hypotension*

In the case of hemorrhagic shock, restoration of tissue perfusion must be balanced against exacerbation of ongoing hemorrhage by increasing blood pressure. This dilemma has given rise to the debate regarding *permissive hypotension*, which involves deliberate tolerance of lower mean arterial pressures in the face of hemorrhagic shock in order to minimize further bleeding. This strategy is based on the notion that decreasing perfusion pressure will maximize success of the body's natural mechanisms for hemostasis, such as arteriolar vasoconstriction, increased blood viscosity, and in situ thrombus formation. Animal models of uncontrolled hemorrhage have revealed that crystalloid resuscitation to either replace three times the lost blood volume (Bickell et al. 1991) or maintain 100 % of pre-injury cardiac output (Owens et al. 1995) exacerbates bleeding (Bickell et al. 1991; Owens et al. 1995) and increases mortality (Bickell et al. 1991) as compared to more limited fluid resuscitation.

Randomized trials that compare fluid management strategies prior to control of hemorrhage among human subjects are limited. In the first large scale trial, Bickell et al. randomized 598 patients in hemorrhagic shock (systolic blood pressure $<$90 mm Hg) who had sustained penetrating torso trauma to either crystalloid resuscitation or no resuscitation prior to operative intervention (Bickell et al. 1994). Pre-specified hemodynamic targets were not used. Mean systolic arterial blood pressure was significantly decreased upon arrival to the emergency department for the delayed resuscitation group as compared to the immediate

resuscitation group (72 mm Hg vs. 79 mm Hg, respectively, $p = 0.02$) with a corresponding increase in survival (70 vs. 62 %, respectively, $p = 0.04$). A trend towards a decreased incidence of postoperative complications was also observed for the delayed resuscitation group.

Two more recent trials have failed to replicate these findings. Turner et al. randomized 1,306 trauma patients with highly diverse injury patterns and levels of stability to receive early vs. delayed or no fluid resuscitation (Turner et al. 2000). Although no mortality difference was observed (10.4 % for the immediate resuscitation group vs. 9.8 % for the delayed/no resuscitation group), protocol compliance was poor (31 % for the early group and 80 % for the delayed/no resuscitation group), limiting interpretability. Most recently, Dutton et al. randomized 110 trauma patients presenting in hemorrhagic shock (systolic blood pressure <90 mm Hg) to receive crystalloid resuscitation to a systolic blood pressure of >70 mm Hg vs. >100 mm Hg (Dutton et al. 2002). Randomization occurred following presentation to the emergency department. Not all patients required operation, and hemorrhage control was determined at the discretion of the trauma surgeon or anesthesiologist. Although there was a significant difference in mean blood pressure during bleeding between the conventional and low groups (114 mm Hg vs. 110 mm Hg, respectively, $p < 0.01$), the mean blood pressure was substantially higher than intended (<70 mm Hg) for the low group, and the absolute difference between group was likely insignificant clinically. Mortality was infrequent and did not vary by resuscitation arm (7.3 % for each group).

Methodological variability between these trials has precluded a meaningful meta-analysis (Kwan et al. 2003), and may help to explain the discrepant mortality findings. It is clear that the degree of hemorrhagic shock was most pronounced in the study of Bickell et al., as evidenced by the lowest presenting systolic blood pressure as well as the highest mortality. Furthermore, randomization was accomplished in the pre-hospital setting, and all patients required operative intervention. By contrast, mortality was infrequent in the study of Dutton et al., and the target systolic blood pressure of 70 mm Hg in the "low" group was, on average, not achieved. Thus, at present, it is possible to conclude that limited volume resuscitation prior to operative intervention may be of benefit among patients with penetrating trauma in hemorrhagic shock, although the optimum level of permissive hypotension remains unknown. The benefit of such therapy among a more diverse cohort of patient in hemorrhagic shock, with a low associated risk of death, is not clear. Finally, regardless of therapeutic benefit, reliable achievement of permissive hypotension appears challenging once hospital care has begun.

### *5.3.3 Resuscitation to Supra-Normal Physiology*

Once definitive hemorrhage control has been obtained, is there benefit to pushing tissue perfusion to a "supra-normal" level? At the cellular level, shock is characterized by oxygen demand that exceeds supply, such that $VO_2$ is $DO_2$-dependent.

Replacement of the oxygen debt is thus signaled by an increase in $DO_2$ that does not result in a corresponding increase in $VO_2$. Shoemaker et al. observed that survivors of shock demonstrate increases in $DO_2$ to "supranormal" levels ($\geq$600 mL/min) as compared to non-survivors (Bland et al. 1985; Velmahos et al. 2000), suggesting that endogenous eradication of the oxygen debt via enhanced physiology is advantageous. This observation led to the hypothesis that resuscitation to supranormal physiology would result in improved outcomes. Thresholds of supranormal physiology have included cardiac index >4.5 L/min/$m^2$, $DO_2$ index >600 mL/min/$m^2$, and $VO_2$ index of >170 mL/min/$m^2$. Unfortunately, results of randomized trials comparing supranormal to conventional resuscitation among critically ill patients have been in large part disappointing (Gattinoni et al. 1995; Velmahos et al. 2000; McKinley et al. 2002; Sandham et al. 2003; Heyland et al. 1996; Richard et al. 2003; Rhodes et al. 2002).

Several possibilities may explain the lack of benefit observed in these trials. As mentioned previously, the timing of goal-attainment is of importance; resuscitation to supranormal physiology following the onset of organ failure does not appear to impart a survival advantage (Kern and Shoemaker 2002). However, increasing $DO_2$ to $\geq$600 mL/min *prior* to the onset of critical illness did not result in a mortality benefit in a randomized trial of nearly 2,000 high-risk surgical patients (Sandham et al. 2003), questioning the findings of the meta-analysis by Kern and Shoemaker. Furthermore, both shock and resultant organ failure are seldom predictable events, thus precluding pre-emptive resuscitation to supranormal hemodynamics.

A second finding in the meta-analysis by Kern and Shoemaker was that the benefits of supranormal resuscitation were observed only if the stated goals were in fact obtained. Although this last point appears intuitive, it is important to note that resuscitation to supranormal physiology is often times impossible. In a recent cohort study of hemorrhagic shock, only 70 % of patients were able to achieve supranormal physiologic values of cardiac index >4.5 L/min/$m^2$, $DO_2$ index >600 mL/min/$m^2$, and $VO_2$ >170 mL/min/$m^2$ (Velmahos et al. 2000). Similarly, pre-specified goals were attained on average in only 7 of 21 (33.3 %) studies included in the meta-analysis by Kern and Shoemaker (Kern and Shoemaker 2002). Beyond impracticality, measurement of the parameters necessary to calculate $DO_2$ and $VO_2$ require a functional pulmonary artery catheter. Both misinterpretation of data (Iberti et al. 1990) and associated morbidity of this device (Ivanov et al. 2000) must be taken into account. Although newer arterial catheter-based calculations of cardiac output have become common (McGee et al. 2007), this modality has not been validated within the context of a goal-directed resuscitation protocol. Perhaps most concerning is the fact that resuscitation to supranormal physiology results invariably in increased volume expansion, thereby increasing the risks of pulmonary edema, intestinal ischemia, and ACS (McKinley et al. 2002; Balogh et al. 2003). In light of these data, resuscitation of shock based on attainment of supra-normal physiology cannot be advocated currently.

### 5.3.4 Red Blood Cell Transfusion

Although the risks and benefits of allogeneic RBC transfusion are discussed elsewhere in this book, we offer some salient points with respect to resuscitation of hemorrhagic shock. Because hemorrhagic shock involves impaired tissue perfusion due to both hypovolemia and anemia, it is intuitive that restoration of the hemoglobin concentration via RBC transfusion both reverses shock and improves outcomes. However, the optimal target hemoglobin concentration during resuscitation remains unknown.

During resuscitation, a balance must occur between the competing goals of maximal oxygen content (hematocrit = 100 %) and minimal blood viscosity (hematocrit = 0 %). Furthermore, irrespective of hematocrit, the oxygen carrying capacity of transfused allogeneic erythrocytes is impaired due to storage-induced changes in both deformability and hemoglobin oxygen affinity. Accordingly, although many studies have measured an increase in $DO_2$ following transfusion of allogeneic RBCs, almost none have reported an increase in $VO_2$ (Napolitano et al. 2009). Finally, beyond a role in $DO_2$, erythrocytes are integral to hemostasis via their involvement in platelet adhesion and activation, as well as thrombin generation. The hematocrit is thus relevant to hemorrhagic shock as it relates to both oxygen availability and hemostatic integrity.

Early canine models of hemorrhagic shock suggested that $VO_2$ is optimized at a relatively high hematocrit (range 35–42 %) (Crowell et al. 1959). However, hematocrit variation was achieved via auto-transfusion of the animal's shed whole blood, eliminating the aforementioned limitations of allogeneic erythrocytes, and rendering the results inapplicable to modern resuscitation of hemorrhagic shock. Furthermore, acute normovolemic hemodilution of dogs to a hematocrit of 10 % is well tolerated, with little decrement in oxygen delivery secondary to a compensatory increase in cardiac output (Takaori and Safar 1966).

Retrospective observations among critically ill surgical patients in the 1970s suggested a hematocrit of 30 % as optimal for both oxygen carrying capacity and survival (Czer and Shoemaker 1978). Such studies formed the basis of the traditional recommendation to maintain the hematocrit >30 %, although the marked limitations of this retrospective literature were recognized ultimately. As the deleterious effects of RBC transfusion became increasingly evident, renewed interest in the ideal transfusion trigger occurred. The Transfusion Requirements in Critical Care (TRICC) Trial, which compared restrictive (hemoglobin <7.0 g/dL) and liberal (hemoglobin <9.0 g/dL) transfusion triggers among 838 patients, provided the first level I evidence regarding RBC transfusion strategies among the critically ill (Hebert et al. 1999). Although inclusion criteria did not specify ongoing resuscitation, 37 % of patients were in shock at the time of enrollment as evidenced by the need for vasoactive drugs. No difference in 30-day mortality was observed between groups. However, in hospital mortality, as well as mortality among less severely ill patients (Acute Physiology and Chronic Health Evaluation II Score <20) and younger patients (age < 55 years) was significantly lower in the

restrictive transfusion group. Current evidence thus suggests that a hemoglobin concentration of >7 g/dL is at least as well tolerated as a hemoglobin concentration of >9 g/dL among critically ill patients, although extrapolation of these data is necessary to extend this recommendation to patients in hemorrhagic shock.

It is possible that hemoglobin concentrations below 7 g/dL are safe, particularly in younger patients. However, a hemoglobin concentration of 5 g/dL appears to be the threshold for critical anemia. Whereas hemodilution of healthy volunteers as low as a hemoglobin concentration of 5 g/dL is well tolerated (Weiskopf et al. 1998), a study of postoperative patients who refused RBC transfusion reported a sharp increase in mortality below this same hemoglobin concentration (Carson et al. 2002). Such populations differ fundamentally from the multiply-injured, exsanguinating patient in need of resuscitation. However, these data are provocative, and future large scale trials of lower transfusion triggers for the resuscitation of hemorrhagic shock are warranted in light of the accumulating evidence documenting the untoward effects of RBC transfusion.

In addition to oxygen transport, RBCs play an important role in hemostasis. As the hematocrit rises, platelets are displaced laterally towards the vessel wall, placing them in contact with the injured endothelium; this phenomenon is referred to as margination. Platelet adhesion via margination appears optimal at a hematocrit of 40 % (Goldsmith 1972). Erythrocytes are also involved in the biochemical and functional responsiveness of activated platelets. Specifically, RBCs increase platelet recruitment, production of thromboxane B2, and release of both ADP and P-thromboglobulin. Furthermore, RBCs participate in thrombin generation through exposure of procoagulant phospholipids. Interestingly, animal models suggest that a decrease of the platelet count of 50,000 is compensated for by a 10 % increase in hematocrit (Quaknine-Orlando et al. 1999). Despite these experimental observations, no prospective data exist detailing the relationship between hematocrit, coagulopathy, and survival among critically injured trauma patients.

The myriad risks of RBC transfusion must be kept in mind during resuscitation of patients in hemorrhagic shock. The immunomodulatory properties of RBC transfusion were first noted as a correlation between transfusion and graft survival following solid organ transplantation (Opelz and Terasaki 1978). The observation that tumor recurrence was associated with RBC transfusion soon followed (Gantt 1981). It is now appreciated that RBC transfusion both impairs humoral immunity and causes elaboration of pro-inflammatory cytokines (Shanwell et al. 1997). These phenomena are both transfusion dose and age dependent. Moreover, transfused blood exerts a number of negative effects upon cardiodynamics, including increased pulmonary vascular resistance, depletion of endogenous nitric oxide stores, and both regional and systemic vasoconstriction (Fernandes et al. 2001).

In summary, prior investigations into the ideal hematocrit for oxygen carrying capacity during hemorrhagic shock are in large part irrelevant to modern day resuscitation with allogeneic blood. Banked erythrocytes are subject to a time dependent diminution of oxygen carrying capacity, and the effect of blood transfusion on oxygen consumption, regardless of hematocrit, remains questionable.

The CRIT trial suggested that patients in shock tolerate a hemoglobin concentration of 7.0 g/dL at least as well as 9.0 g/dL, although this hypothesis was not tested during the initial resuscitation of hemorrhagic shock specifically. Furthermore, the role of erythrocytes in hemostasis must be considered. In practice, clinical circumstance (e.g., ongoing hemorrhage with hemodynamic instability and coagulopathy), as opposed to an isolated laboratory measurement, should inform the decision to transfuse. However, until there is definitive evidence to challenge the CRIT data, a hemoglobin concentration of <7 g/dL should be considered the default transfusion trigger for resuscitation from shock.

One final point regarding the use of blood products for the resuscitation of patients in hemorrhagic shock involves the relative quantities of RBCs, clotting factors, and platelets to administer. It is now recognized that resuscitation of patients who require massive transfusion (>10 U RBC within 24 h) using RBCs alone results in coagulopathy due to both consumption and dilution of coagulation factors as well as platelets. Both acidosis and hypothermia exacerbate this coagulopathy. Our group and others noted that mortality among massively transfused patients was reduced when increased amounts of both plasma and platelets were administered empirically. Specifically, when introducing the concept of RBC:FFP ratios, we reported increased mortality among a cohort of patients with major vascular trauma associated with RBC:FFP ratios greater than 5:1, with overt coagulopathy observed nearly universally with ratios exceeding 8:1 (Kashuk et al. 1982). In 2007, Borgmen et al. published a series of 254 massively transfused US soldiers in Iraq and Afganistan, reporting markedly improved survival among those transfused with a RBC:FFP ratio in the range of 1.5:1, as compared to higher ratios.(Borgman et al. 2007) This ratio appeared appealing intuitively as it most closely resembled that of whole blood, although a 1:1 formulation is actually both anemic (hematocrit 27 %) and clotting factor deficient (65 % activity) as compared to fresh whole blood (Armand and Hess 2003). Several subsequent studies, in both the military and civilian setting, have corroborated the findings of Borgeman et al. (Duchesne et al. 2008; Holcomb et al. 2008; Teixeira et al. 2009). An association between early, aggressive FFP administration and improved survival has also been documented among trauma patients who underwent sub-MT (Spinella et al. 2008). These data have given rise to the concept of *damage control resuscitation*, which involves early transfusion of increased amounts of both clotting factors and platelets, in addition to minimization of crystalloid resuscitation in patients who are expected to require MT. Currently, many trauma centers advocate pre-emptive transfusion of RBC:FFP using a target ratio of 1:1 for such patients.

Unfortunately, the literature addressing component transfusion ratios during MT suffers from several substantial methodological limitations. Despite a myriad of retrospective data, mathematical models (Hirshberg et al. 2003), and expert opinion, there remains no prospective evidence to support an empiric transfusion ratio. A major limitation of the retrospective literature involves survival bias. Specifically, it remains unclear if increased FFP transfusion improves survival or if patients who survive simply live long enough to receive more FFP. Indeed,

patients who are bleeding faster get less plasma as the trauma team and blood bank struggle to keep up. Related intimately to the issue of survival bias is that of the time period over which the RBC:FFP ratio is calculated. Although over 80 % of RBC transfusions are administered within 6 h of injury, most studies have reported the cumulative RBC:FFP ratio as calculated at 24 h. Such a strategy exacerbates survival bias, as the RBC:FFP ratio is known to decrease over time. Accounting for the time-dependent nature of the RBC:FFP transfusion ratio eliminated any association with survival in one recent report (Snyder et al. 2009). Furthermore, when the cumulative RBC:FFP ratio was analyzed at 6 h as opposed to 24, our group identified a ratio in the range of 2:1–3:1, as opposed to 1:1, as associated with the lowest predicted mortality (Kashuk et al. 2008).

The next major limitation involves the lack of a mechanistic link between a lower RBC:FFP ratio and improved survival. The clinical efficacy of FFP remains largely unproven (O'Shaughnessy and Atterbury 2004), and no study has documented an association between a lower RBC:FFP ratio and fewer total blood products administered. Moreover, differences in laboratory markers of coagulopathy (e.g., PT, TEG) have not been demonstrated between groups of varying RBC:FFP ratios. In fact, a canine model showed no benefit to adding FFP following MT in terms of changes in coagulation protein levels or clotting times (Martin et al. 1985). Finally, the benefit of a 1:1 RBC:FFP ratio has also not been consistent across various mechanisms of injury (Mace et al. 2009).

Although many experts advocate a RBC:FFP transfusion ratio of 1:1 during MT, the lowest ratio achieved in most studies approaches 1.5:1. While moving from an RBC:FFP ratio of 2:1–1:1 may appear trivial, such a paradigm shift represents a 100 % increase in FFP utilization. An increase of this magnitude would place tremendous strain upon the marginal FFP donor pool, as well as increase exponentially blood bank labor, likely to the point of non-sustainability in the event of a mass casualty. Finally, unbridled FFP administrated must be viewed with caution in light of the accumulating evidence detailing the immunomodulatory properties of such therapy.

In summary, the literature involving empiric component therapy suffers from several methodological limitations. Currently, the optimal empiric RBC:FFP ratio for resuscitation of patients who require MT appears to be in the range of 1:2–s1:3. However, whenever possible, component replacement should be both individualized and goal-directed, such that overzealous clotting factor and platelet replacement and the complications thereof are minimized.

## 5.4 Complications of Resuscitation

Resuscitation beyond restoration of adequate tissue perfusion is both ineffective and potentially harmful. Although aggressive volume expansion may be lifesaving for the immediate resuscitation of severe hypotension, subsequent fluid administration should occur only when evidence of preload responsiveness exists

(Vincent and Weil 2006). Excessive volume expansion is particularly detrimental in the setting of critical illness as inflammatory-mediated increased capillary permeability drives fluid from the intravascular space into the interstitium, compounding tissue edema with little to no improvement in hemodynamics. The effects of tissue edema on specific organ systems are summarized in Table 5.3.

Intra-abdominal hypertension leading to *abdominal compartment syndrome* (ACS) is a particularly devastating complication of volume resuscitation with a high associated morbidity and mortality (Cheatham et al. 2007). Once believed to be a complication specific to trauma patients, both intra-abdominal hypertension and ACS have now been reported over a wide variety of patient cohorts, ranging from organ transplantation (Biancofiore et al. 2003) to pediatrics (Beck et al. 2001; Ball et al. 2008). The pathophysiology of ACS involves a progressive increase in abdominal pressure due to any combination of diminished abdominal wall compliance, increased intra-luminal intestinal contents, increased intra-peritoneal fluid, and increased tissue edema. Importantly, the abdomen need not be the initial location of the pathology (Madigan et al. 2008); the development of ACS in this instance is termed *secondary ACS*. Increases in abdominal pressure eventually become sufficient to impede venous return from both abdominal viscera (resulting in intestinal ischemia) and the inferior vena cava (causing decreased filling pressures and obstructive shock). Both impedance of urinary drainage and respiratory embarrassment secondary to elevated airway pressures are also characteristic. Several risk factors for ACS are recognized; both large volume fluid resuscitation and attempts to resuscitate to supranormal physiology have been implicated consistently (Balogh et al. 2003; Madigan et al. 2008; Malbrain et al. 2005).

Physical exam findings, such as elevated airway pressures, oliguria, and tube feeding intolerance, may aid in the diagnosis of abdominal hypertension, but are in and of themselves insensitive (Kirkpatrick et al. 2000; Greenhalgh and Warden 1994), mandating measurement of intra-abdominal pressure. Several techniques have been described, including transduction of intra-gastric, intravascular, and intraperitoneal pressure. Measurement of the intravascular pressure is the current reference standard with several noteworthy technical considerations. Pressure is expressed as mm Hg. Normal intra-abdominal pressure is $<7$ mmHg, increases $>12$ mmHg constitute abdominal hypertension, and a sustained pressure $\geq 20$ mm

**Table 5.3** Effects of tissue edema on specific organ systems

| Organ system | Pathology |
|---|---|
| Central nervous | Elevated intra-cranial pressure |
| Pulmonary | Hypoxia, decreased compliance |
| Cardiovascular | Increased afterload, subendocardial ischemia, |
| Renal | Decreased re-absorption, impaired concentrating ability |
| Gastrointestinal | Feeding intolerance, adynamic ileus, abdominal compartment syndrome |
| Integument, musculoskeletal | Skin ulcerations, breakdown, muscle compartment syndrome |

Hg in the presence of organ failure is diagnostic of ACS. Disease severity may also be expressed as the abdominal perfusion pressure, defined as the mean arterial pressure minus the intra-abdominal pressure. An abdominal perfusion pressure <50–60 mmHg is associated with poor outcomes among patients with intra-abdominal hypertension (Cheatham et al. 2000).

Medical therapy aimed at reducing abdominal pressure may be attempted for the hemodynamically stable patient in the absence of worsening organ failure. Paralysis, intestinal decompression, and diuresis are all effective means to decrease abdominal pressure. However, sustained or worsening intra-abdominal hypertension after a brief trial of non-operative maneuvers mandates surgical decompression, as delay in definitive decompression worsens outcomes substantially (Cheatham et al. 2000). Percutaenous catheter decompression may be considered when elevated abdominal pressure is secondary to intra-peritoneal fluid (e.g., ascites). Small case series suggest that this technique may be particularly useful among burn patients (Latenser et al. 2002; Corcos and Sherman 2001; Parra et al. 2006). However, beyond this specific circumstance, surgical decompression via laparotomy remains the definitive treatment for ACS. Failure of improvement following surgical decompression should raise concern for either inadequate decompression or misdiagnosis. When timely and effective surgical decompression is achieved, the abdominal is usually amenable to closure within 7 days (Burlew et al. 2012).

Beyond ACS, aggressive volume expansion results in a variety of additional untoward consequences. Positive fluid balance is associated with worsened pulmonary dynamics among patients with acute lung injury (Wiedemann et al. 2006; Sakr et al. 2005) as well as increased post-operative complications following gastrointestinal surgery (Brandstrup et al. 2003; Kudsk 2003; Nisanevich et al. 2005). Importantly, intentional fluid restriction during critical illness does not appear to increase the incidence of renal injury given that it is monitored vigilantly (Wiedemann et al. 2006). Although the ideal fluid management strategy both during and following resuscitation remains controversial (Bagshaw and Bellomo 2007; Pruitt 2000; Durairaj and Schmidt 2008), recent evidence suggests that restrictive fluid strategies are well tolerated, and may even be beneficial. The use of dynamic measurements of fluid responsiveness (Marik et al. 2009), restrictive transfusion triggers (Hebert et al. 1999), and avoidance of routine supra-physiologic resuscitation (Balogh et al. 2003) represent evidence-based strategies to minimize unnecessary and potentially harmful volume expansion.

## 5.5 Summary

Effective resuscitation of patients in hemorrhagic shock entails balancing timely restoration of tissue perfusion with parsimonious subsequent volume expansion in order to avoid the detrimental effects of fluid overload. Although definitive evidence documenting a benefit to permissive hypotension is lacking, periods of

hypertension should be avoided in the bleeding patient, and definitive hemorrhage control should be obtained immediately. When diagnosing shock and monitoring resuscitative efforts, dynamic measurements of fluid responsiveness are preferred, and endpoints of resuscitation should incorporate downstream markers that normalize rapidly following restoration of tissue perfusion (e.g., $SvO_2$, base deficit). The target hemoglobin concentration during resuscitation of patients in hemorrhagic shock remains unknown, although it is likely somewhere between 7 and 10 mg/dL. Rather, hemodynamic instability in the face of ongoing hemorrhage should prompt additional transfusion. Current evidence suggests replacing RBCs and FFP in a ratio of 2:1. Resuscitation of patients in hemorrhagic shock invariably involves massive volume expansion; knowledge of potential complications, particularly ACS, is imperative for any clinician caring for these patients.

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# Chapter 6
# Allogeneic Blood Transfusion for Surgical and Traumatic Hemorrhage

**Mercy Kuriyan and Jeffrey L. Carson**

## 6.1 Introduction

Hemorrhage in surgical and trauma patients can lead to shock, which occurs due to the inadequacy of the circulatory system to adequately perfuse tissues and meet oxygen demand. Surgical control of bleeding, fluid resuscitation and blood transfusions are interventions used to treat bleeding (Tien et al. 2007). Resuscitation with fluids and red blood cells are given with the goal of improving perfusion and oxygen delivery.

In this chapter we review studies evaluating impact of red blood cell transfusion on clinical outcomes. We begin by describing the risks associated with anemia. We then summarize the clinical trial evidence on red cell transfusion thresholds based on a systematic Cochrane review (Carson et al. 2012; Carson et al. 2013). We limit our review to clinical trials because observational studies evaluating transfusion thresholds are subject to uncontrolled confounding (Middelburg et al. 2010). We complete the chapter with summary of clinical guidelines.

## 6.2 Red Cell Transfusion Goal/Rationale

Oxygen delivery to the tissues depends upon the concentration of hemoglobin, the percent saturation of that hemoglobin, and the cardiac output. A reduction in oxygen delivery below a critical level deprives tissues of the oxygen necessary for

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oxidative metabolism. Maintaining adequate oxygen delivery may result in improved clinical outcome since oxygen requirement by tissues may be increased in acute stress situations.

Increasing hemoglobin level is thought to increase oxygen delivery. Clinical studies show however that when isovolemic hemodilution occurs the oxygen extraction ratio increased as hematocrit decreased. With progressive increases in hematocrit, oxygen delivery increased (Mathru et al. 1991). Until recently, there has been a lack of clarity as to the decision to transfuse RBCs to patients undergoing surgery, anesthesia and in critically ill patients.

## 6.3 Risk of Anemia

Several studies have examined the risks associated with different levels of anemia. The effects of isovolemic anemia was evaluated in a series of experimental studies in healthy volunteers. In 55 volunteers, reversible ST segment depression developed in 3 subjects at hemoglobin concentration of 5 to 7/g dL (Weiskopf et al. 1998; Leung et al. 2000). In nine subjects with mean age of 25, cognitive changes began between 5 to 6 g/dL and resolved when hemoglobin level returned to 7 g/dL (Weiskopf et al. 2000). Whether these results would apply to older surgical patients is unknown.

A retrospective cohort study examined the risks associated with anemia in 1958 patients, 18 years and older, who underwent surgery and declined blood transfusion for religious reasons (Carson et al. 1996). Decline in hemoglobin which is the difference between preoperative and postoperative hemoglobin concentrations was used to estimate blood loss. The mortality was 1.3 % (95 % confidence interval, 0.8–2.0) in patients with preoperative hemoglobin 12 g/dL or greater and 33.3 % (95 % confidence interval, 18.6–51.0) in patients with preoperative hemoglobin less than 6 g/dL. The increase in risk of death associated with low preoperative hemoglobin was more pronounced in patients with cardiovascular disease than in patients without cardiovascular disease (interaction $p < 0.03$) and in those with greater blood loss (Fig. 6.1), (Carson et al. 2012). These data suggest that patients with cardiovascular disease may be less tolerant of anemia than those without the disease.

The risk of death was further evaluated in a subgroup of 300 these patients with postoperative hemoglobin less than 8 g/dL (Carson et al. 2002). In patients with a postoperative hemoglobin concentration between 7.1 and 8.0 g/dL, none of the patients died. In patients with postoperative hemoglobin level between 5.1 and 7.0 g/dL, about 9 % of patients died, and at 5.0 g/dL or lower, mortality was very high. Overall, the odds of death in patients with a postoperative hemoglobin level $\leq$ 8 g/dL increased 2.5 times (95 % confidence interval, 1.9–3.2) for each gram decrease in hemoglobin level.

The association of preoperative anemia and 30 day postoperative mortality was evaluated in a cohort study of 310,000 VA patients 65 years age or older undergoing non cardiac surgery. Mortality rose by 1.6 % for every 1 % level of hematocrit below normal (Wu et al. 2007). Similarly, a retrospective study based

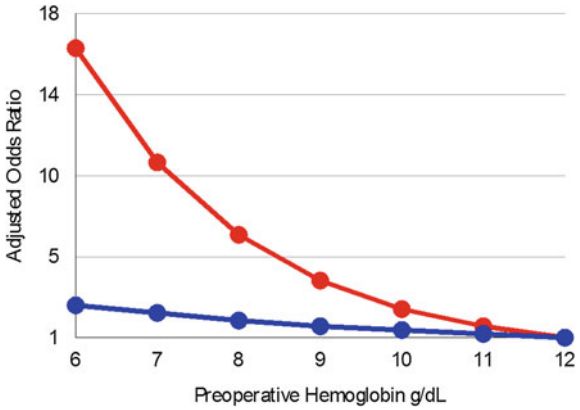


**Fig. 6.1** Adjusted odds of death stratified by presence of cardiovascular (*red*) compared to patients without cardiovascular disease (*blue*) (Carson et al. 1996)

on 17,056 consecutive cardiac surgery patients showed that anemic patients had a significantly higher rate of stroke (1 % vs. 0 %, p = 0.045), major morbidity (27.4 vs. 17.5 %, p = 0.001), and a higher operative mortality rate (12.7 vs. 7.5 %, p = 0.014) (Ranucci et al. 2012).

## 6.4 Clinical Trials

A systematic review of the literature identified 19 clinical trials that evaluated liberal versus restrictive transfusion thresholds (Carson et al. 2012) (Fig. 6.2). We are aware of two additional small pilot trials that have been published (Cooper et al. 2011; Shehata et al. 2012). Three trials will be described in detail.

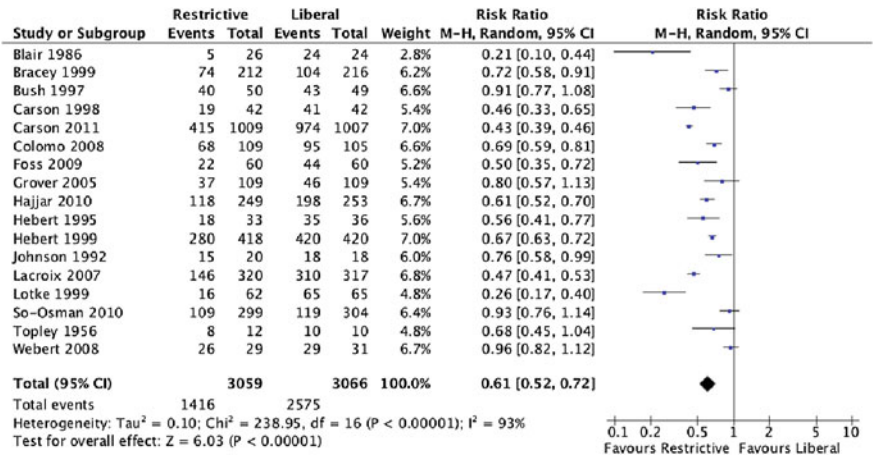


**Fig. 6.2** Clinical trials comparing liberal versus restrictive transfusion thresholds and risk of transfusion exposure to blood transfusion (Carson et al. 2012)

The landmark clinical trial in the field of transfusion threshold research is the Transfusion Requirement in Critical Care trial (TRICC) that was conducted in adult intensive care unit patients (Hebert et al. 1999). Euvolemic intensive unit patients were randomly allocated to 10 g/dL (liberal) transfusion group and 7 g/dL (restrictive) transfusion group. Thirty-day mortality was lower in patients in the 7 g/dL transfusion group (18.7 %) than the 10 g/dL group (23.3 %), although the results were not statistically significant ($p = 0.1$). The restrictive transfusion group had fewer myocardial infarctions ($p = 0.02$), pulmonary edema ($p = 0.01$), and acute respiratory distress syndrome ($p = 0.06$) than 10 g/dL group. In the subgroup of patients with ischemic heart disease, however, the mortality rate was nonsignificantly ($p = 0.3$) higher in the restrictive group (26 %) compared to liberal group (21 %). The TRICC trial included a mix of surgical (25 %), trauma (20 %), and medical patients.

The Transfusion Requirements After Cardiac Surgery (TRACS) study is a single center clinical trial conducted in Brazil in 502 patients who had cardiac surgery (Hajjar et al. 2010). Liberal transfusion group received transfusion if hematocrit was less than 30 % and the restrictive transfusion group if hematocrit was less than 24 %. The primary outcome was the composite of 30-day all-cause mortality and morbidity including cardiogenic shock, acute respiratory distress syndrome, or acute renal injury requiring dialysis or hemofiltration) occurring during the hospital stay. The primary outcome (11 %- restrictive versus 10 %- liberal) and secondary outcomes were similar in the two transfusion groups. Another trial in cardiac surgery compared 8 g/dL threshold to 9 g/dL threshold in 428 patients and found no difference in outcomes (Bracey et al. 1999).

The Transfusion Trigger Trial for Functional Outcomes in Cardiovascular Patients Undergoing Surgical Hip Fracture Repair (FOCUS) is a randomized clinical trial conducted in 47 centers in the US and Canada that compared to two transfusion thresholds (Carson et al. 2011). 2016 patients (mean age 81) with cardiovascular disease or risk factors who received surgical repair of a fractured hip were randomly allocated to receive either enough blood to raise and maintain hemoglobin concentrations at 10 g/dL or greater or to receive red cell transfusions when symptoms of anemia developed or if hemoglobin concentration dropped below 8 g/dL. There was no difference in the primary outcome of death or inability to walk across a room unassisted, 35.2 %- liberal group and 34.7 %- restrictive group. There were also no differences in secondary outcomes including 30-days mortality (liberal = 5.2 % vs. restrictive = 4.3 %) and 60-day mortality (liberal = 7.6 % vs. restrictive = 6.6 %) pneumonia, function scores, or fatigue. The primary cardiac outcome of in-hospital acute coronary syndrome or death occurred in 4.3 % of the liberal group, and 5.2 % of the restrictive group (difference −0.9 %; 99 % confidence interval −3.3 % to 1.6 %). Myocardial infarction occurred less commonly in the liberal group (2.3 %) than the restrictive group (3.8 %) although the wide confidence intervals are consistent with an underpowered comparison (odds ratio and 99 % confidence interval, 0.60; 0.30 to 1.19 and absolute difference −1.5 %, 99 % confidence interval, −3.5 to 0.5). FOCUS provided clinical trial evidence that patients with pre-existing cardiovascular disease can be safely managed using a restrictive transfusion strategy.

Most of the remaining trials were small and underpowered in adults or were in pediatric ICU patients (Lacroix et al. 2007).

Since the publication of systematic review, an important trial has been published.(Villanueva et al 2013 [1]) Patients with severe upper gastrointestinal bleeding wererandomly allocated to restrictive (7 g/dL) transfusion threshold or liberal (9 g/dL)threshold. Patients were excluded with massive exsanguinating bleeding, acute coronary syndrome, or other evidence of vascular disease. At 45 days post randomization, mortality was lower in the restrictive group (5%) than liberal group (9%); p=0.02. Recurrent bleeding and portal pressures were lower in the 7 g/dL group than the 9 g/dL group.

## 6.5 Meta-Analysis of Clinical Trials

A total of 19 randomized clinical trials published up to 2011 including 6264 patients were included in meta-analysis (Carson et al. 2012) (Fig. 6.2). The systematic review only included trials and not observational studies that are subject to bias. Eight studies were in surgery patient (cardiac, vascular or orthopedic), (Hajjar et al. 2010; Bracey et al. 1999; Carson et al. 2011; Bush et al. 1997; Carson et al. 1998; So-Osman et al. 2010; Foss et al. 2009; Grover et al. 2006; Johnson et al. 1992; Lotke et al. 1999) five in patients with acute blood loss and/or trauma (Lacroix et al. 2007; Blair et al. 1986; Colomo et al. 2009; Fortune et al. 1987; Topley and Fischer 1956), and three in patients treated in critical care units (Hebert et al. 1999; Lacroix et al. 2007; Hebert et al. 1995).

Despite significant heterogeneity in the types of patients included in the trials, the results of the trials were similar. (Carson et al. 2012) Restrictive transfusion strategies reduced the risk of receiving a red blood cell (RBC) transfusion by 39 % (RR = 0.61; 95 % CI 0.52–0.72) and the number of units by 1.2 units (95 % CI 0.53–1.85 units), (Fig. 6.2). Importantly, restrictive transfusion strategies did not appear to be harmful. The risk of mortality, cardiac events, myocardial infarction, stroke, pneumonia and thromboembolism were not elevated in restrictive transfusion group compared to the liberal transfusion group. Restrictive transfusion strategies were associated with a statistically significant reduction in hospital mortality (relative risk = 0.77; 95 % confidence interval, 0.62–0.95) but not 30-days mortality (relative risk = 0.85; 95 % CI 0.70–1.03), (Fig. 6.3). Also, functional recovery and length of hospital stay was not adversely impacted by restrictive transfusion.

## 6.6 Transfusion Needs in Traumatic Hemorrhage

The goals of treatment in trauma patients include avoiding metabolic acidosis, hypothermia, treating coagulopathy and stabilizing the patient as soon as possible (Theusinger et al. 2012).

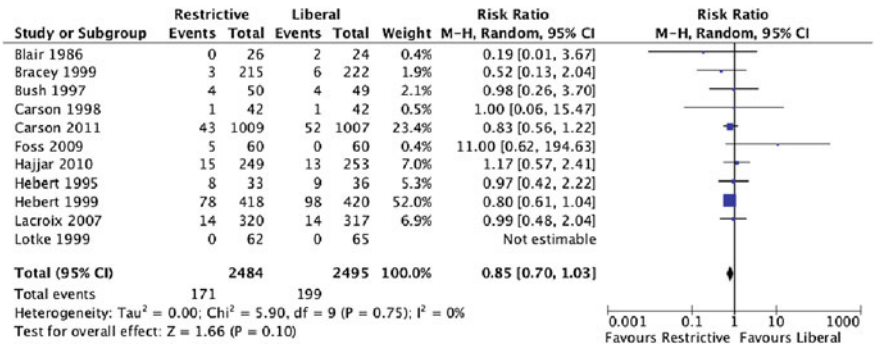


**Fig. 6.3** 30-days mortality in clinical trials comparing liberal versus restrictive blood transfusion strategy (Carson et al. 2012)

Massive hemorrhage protocols are used to avoid delays in the delivery of blood and blood components to patients with hemorrhagic shock. A rapidly emerging trend is to transfuse 1 unit of fresh frozen plasma, 1 unit of platelets for each unit of red blood cells in massive hemorrhaging patient. However, the observational studies that demonstrate improved mortality are subject to bias because patients who are bleeding more slowly had time to get higher volumes of plasma and platelets than patients who were bleeding more quickly. A clinical trial called Pragmatic, Randomized Optimal Platelet and Plasma Ratios (PROPPR) has started enrolling patients and has a target sample size of 580 patients (PROPPR—Pragmatic 2012). Military physicians believe there are distinct advantages in fresh warm whole blood over component therapy during the massive resuscitation of acidotic, hypothermic, and coagulopathic trauma patients (Repine et al. 2006; Spinella et al. 2009). In the civilian population due to logistic and regulatory restrictions this is not a practical approach.

## 6.7 Current Clinical Practice Guidelines

Evidence-based recommendations regarding the use of RBC transfusion in adult trauma and critical care are provided for transfusions in critically ill patients, in sepsis, in those at risk for acute lung injury, acute respiratory distress syndrome and those with neurologic injury or disease (Napolitano et al. 2009). These guidelines recommended a restrictive strategy (transfusion when the hemoglobin level is less than 7 g/dL) for adult trauma and critical care patients, with the exception of those with acute myocardial ischemia. Furthermore, these guidelines recommended avoiding transfusion based only on a hemoglobin trigger. Instead, the decision should be guided by such individual factors as bleeding, cardiopulmonary status, and intravascular volume.

The Red Blood Cell Transfusion clinical practice guideline from the AABB (American Association of Blood Banks) recommends that in surgical patients transfusion should be considered at hemoglobin less than or equal to (7 to 8 g/dL) in stable patients; adhering to a restrictive strategy in hospitalized patients with preexisting cardiovascular disease and considering transfusion for patients with symptoms or a hemoglobin level of 8 g/dL or less (Carson et al. 2012), (Table 6.1). Symptoms as well as hemoglobin concentration should be evaluated during transfusion decisions (Table 6.2). The recommendation after evaluating all available data was that a restrictive transfusion strategy that uses these thresholds should be considered in most patient populations (hemodynamically stable critical care, surgical, and medical) (Carson et al. 2012).

**Table 6.1** Mortality associated with preoperative hemoglobin levels (Carson et al. 1996)

| Preoperative hemoglobin g/dL | N | % Dead | 95 % Confidence interval |
|---|---|---|---|
| 0–5.9 | 36 | 33.3 | 18.6–51.0 |
| 6.0–6.9 | 27 | 18.5 | 6.3–38.1 |
| 7.0–7.9 | 49 | 12.2 | 4.6–24.7 |
| 8.0–8.9 | 39 | 12.8 | 4.3–27.4 |
| 9.0–9.9 | 75 | 8.0 | 3.0–16.6 |
| 10.0–10.9 | 109 | 4.6 | 1.5–10.4 |
| 11.0–11.9 | 212 | 2.4 | 0.8–5.4 |
| 12+ | 1411 | 1.3 | 0.8–2.0 |

**Table 6.2** AABB guidelines on transfusion thresholds (Carson et al. 2012)

| Question | Recommendation |
|---|---|
| In hospitalized hemodynamically stable patients, at what Hgb should a decision to transfuse RBC be considered | We recommend adhering to a restrictive transfusion strategy<br>In adult and pediatric ICU patients, transfusion should be considered at Hgb $\leq$ 7 g/dL<br>In surgical patients, transfusion should be considered at Hgb $\leq$ 8 g/dL or for symptoms |
| In hospitalized hemodynamically stable patients, with pre-existing cardiovascular disease, at what Hgb should a decision to transfuse RBC be considered? | We suggest adhering to a restrictive transfusion strategy<br>Transfusion should be considered at Hgb $\leq$ 8 g/dL or for symptoms |
| In hospitalized hemodynamically stable patients, should transfusion be guided by symptoms rather than hemoglobin concentration? | We suggest that transfusion decisions should be influenced by symptoms as well as hemoglobin concentration |
| In hospitalized hemodynamically stable patients, with acute coronary syndrome, at what Hgb should a decision to transfuse RBC be considered? | We cannot recommend for or against liberal or restrictive transfusion threshold. Further research is needed to determine optimal RBC transfusion threshold |

## 6.8 Summary

Clinical trials in critically ill adult and pediatric patients, elderly surgical patients with underlying cardiovascular disease and patients undergoing cardiac surgery demonstrate that a restrictive transfusion strategy (7–8 g/dL or symptoms) is safe and associated with much less blood use. Whether lower thresholds or transfusion guided only by symptoms can be safely used has not been adequately evaluated and awaits further study. Clinical trials are also needed (but are not limited to) in patients with acute coronary syndrome, elderly medical patients recovering from illnesses that result in hospitalization, patients with gastrointestinal bleeding, transfusion-dependent patients, patients with coagulopathy or hemorrhagic shock, and patients with traumatic brain injury. We recommend that clinicians incorporate results from clinical trials into an individualized assessment of the need for transfusion based on a careful beside evaluation of the patient's co-morbidity, symptoms and signs.

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# Chapter 7
# Pre-Hospital Fluid Resuscitation in Civilian and Military Populations

**Robert M. Van Haren, Chad M. Thorson, Col Lorne H. Blackbourne and Kenneth G. Proctor**

## 7.1 Historical Background

Major Jonathan Letterman was the medical director of the Army of the Potomac and the founding father of military medicine. Letterman organized the first forward first-aid stations, mobile field hospitals, and ambulance services for the evacuation of wounded soldiers during the Civil War (Tooker 2007). The US Army General Hospital at the Presidio of San Francisco was named for him in 1911. Many innovations in civilian trauma care around the world can be traced to this single institution and to ideas that originated from necessity on the battlefield.

By 1918, Letterman Hospital was the Army's largest general hospital and served troops in the western United States and those returning from overseas during World War I. By the mid 50 s, Letterman had trained a quarter of the Army's medical specialists. Staff physicians and scientists pioneered advances in orthopedic devices, the field of physical therapy and combat casualty care research (Delehanty 2012). In addition, one of the first "blood substitutes", or hemoglobin-based oxygen carriers (HBOC), can be attributed to cutting edge research at Letterman (Hess 1996).

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Thought leaders have long recognized that early intervention to stop bleeding and expand volume could save up to 30 % of those who die on the battlefield, that fresh human blood was the ideal resuscitant, and that storage and transport issues were the main barriers to its widespread use (Bellamy 1987). In recent wars, outdating of stored blood resulted in >60 % of units being discarded. For these and other reasons, the military invested tens of millions in the development of HBOCs. By 1993, its final year, the Letterman Army Institute of Research under the direction of COL JR Hess had developed a variety of cell, tissue, organ, and animal systems and had evaluated the production, safety and efficacy of several candidate HBOCs (Hess, Macdonald et al. 1994; Hess and Reiss 1996). By the mid-1990s, a successful partnership had evolved between the military and industry to scale up production and testing, and led to the first prehospital trauma trial of the most promising, first generation HBOC (Bowersox and Hess 1994; Hess 1995). By this point, several other companies were racing to develop their own new and improved alternatives. Unfortunately, those early triumphs have been followed by almost 15 continuous years of disappointments and set-backs.

As of today, a safe effective HBOC remains an attractive, albeit elusive, concept. There is clearly a critical need for a universal emergency resuscitation fluid for patients in severe hemorrhagic shock where blood is not available during prolonged evacuation or transport. The purpose of this report is to identify areas where prehospital resuscitation can improve in the future.

## 7.2 Where are We Today?

The first two HBOC phase III trauma trials (Sloan, Koenigsberg et al. 1999; Moore, Moore et al. 2009) were unsuccessful. Diaspirin cross-linked hemoglobin (DCLHb, HemAssist®, Baxter) was tested in a multicenter, randomized trial of 18 trauma centers with 112 patients. Because of compelling preclinical data and successful human safety trials, and the urgent unmet need in trauma, the FDA granted an exception to informed consent which allowed the trial to commence in 1996. Despite apparent early success, the trial was stopped prematurely in 1998, when higher mortality rates were observed in the DCLHb group (Sloan, Koenigsberg et al. 1999). The second trial was based on the lessons learned from DCLHb. A prospective, multicenter trial began in 2003 at 29 urban level I trauma centers with a planned enrollment of 714 hypotensive trauma patients randomized to receive a second generation HBOC, human polymerized hemoglobin (Poly SFH-P, PolyHeme®, Northfield Laboratories). This trial was also granted an exception to informed consent. It was completed in 2006, but further development was suspended because there was no significant improvement in 30 days mortality, and more adverse events in the Poly SFH-P group (Moore, Moore et al. 2009).

In the early 2000s, the US Navy, in partnership with Biopure Corp, submitted an investigational new drug application (IND) for RESUS (Restore Effective Survival in Shock), a single- blinded, multi-center, randomized, controlled, Phase

IIb/III clinical trial with another HBOC. Patients would be randomized to receive either HBOC-201 (Hemopure® or standard therapy (crystalloid solution) at the scene of the injury and during transport to the hospital. This trial would also require an exception from informed consent and would include a community consultation and disclosure program as defined in the code of federal regulations. Because of the results from the first two trials, the US Food and Drug Administration (FDA) was particularly concerned about the vasoconstrictor actions of all HBOCs as a class. From 2002–2008, several meetings were held between representatives of the Navy, Biopure, FDA, academic trauma experts, and consumer advocates to debate the pros and cons of prehospital treatment of hemorrhagic shock with HBOC. Ultimately, the FDA expressed concern about the risk benefit profile in patients with waived informed consent, and refused to approve the IND for RESUS. In 2009, pursuit of the RESUS trial was halted but HBOC-201 continued to be developed by a successor company (OPK Biotech).

Thus, in 2012, prehospital resuscitation remains fundamentally the same as it was in the year 1831, when intravenous saline solution was first administered to a patient in shock. Today, crystalloid remains the main treatment for prehospital resuscitation in civilians. In the past 180 years, there have been tremendous advancements in every other aspect of life, but medics in both civilian and military settings continue to use an intravenous saline solution that has hardly changed since 1831 (Blackbourne 2011).

On the battlefield, hemorrhagic shock refractory to resuscitation is the leading cause of preventable traumatic death (Heckbert, Vedder et al. 1998). Saline resuscitation alone is inadequate, as recent studies have shown increased rates of multi-organ failure, acute respiratory distress, and abdominal compartment syndrome in patients who receive crystalloid resuscitation in a ratio greater than 1.5:1 per unit of packed red blood cells (PRBCs) transfused (Neal, Hoffman et al. 2012). Also, fluid resuscitation alone or in combination with PRBC will cause dilutional coagulopathy (Holcomb, Wade et al. 2008). There remains a critical need in both civilian and military populations for an universally compatible oxygen carrying fluid when blood is not readily available (Moore, Cheng et al. 2006).

In the military, those who die before receiving emergency first aid are termed KIA (killed in action), while those who die after admission to a hospital are termed DOW (died of wounds). Hemorrhage accounts for about 80 % of the potentially survivable cases who DOW (Eastridge, Hardin et al. 2011). The majority of these fatal wounds were in the torso (48 %), followed by extremity (31 %), and junctional injuries (21 %). Traumatic brain injuries were the leading cause (83 %) of death in those termed nonsurvivable injuries. The large number of military deaths from hemorrhage highlights the importance of effective hemostasis and the unmet need for an improved fluid for prehospital resuscitation. These two areas have the greatest potential to decrease DOW and KIA rates in the military (Blackbourne, Czarnik et al. 2010; Gerhardt 2011).

In most civilian populations, the majority of injury related deaths also occur prehospital (Sauaia, Moore et al. 1995; Soreide, Kruger et al. 2007). For example, in a 7 year retrospective review of the Swedish nationwide hospital discharge

registry of 414,297 serious injuries, the overall risk of prehospital death was 4.2 % compared to 2.8 % for hospital deaths (21). Prehospital deaths were more likely to be male and younger than 64 years old. In fact for patients younger than 64 years, for each hospital death there were 9 prehospital deaths. A high proportion of all patients (67 %) with gunshot wounds (GSW) died prehospital (Gedeborg, Chen et al. 2012). At our trauma center, a high proportion of deaths arrive with no vital signs on admission(Van Haren, Thorson et al. 2012). Altogether, this shows that prehospital hemorrhage control and resuscitation is an area for improvement in civilian populations.

For any HBOC to be successful for military or civilian prehospital resuscitation, it must improve the coagulopathy of trauma, as well as increase tissue oxygen delivery. Correction of coagulopathy is essential because it is an independent predictor of mortality in civilian and military populations (MacLeod, Lynn et al. 2003; Niles, McLaughlin et al. 2008).

## 7.3 Goals of Prehospital Resuscitation

The two main goals of prehospital resuscitation are to (1) stop hemorrhage and (2) maintain/restore energy production. Hemorrhage control can be achieved manually or with the assistance of hemostatic agents. In general, principles of Advanced Trauma Life Support (ATLS) and Prehospital Trauma Life Support manual guidelines are followed such as primary (Airway, Breathing, Circulation, Disability, Exposure) and secondary surveys for civilian and military populations, respectively. However, a detailed description of hemorrhage control is beyond the scope of this chapter. At the cellular level, energy production is achieved by aerobic metabolism producing adenosine triphosphate (ATP). This is dependent on the delivery of oxygen to the tissue, which requires a sufficient supply of red blood cells (McSwain, Champion et al. 2011). Until hemorrhage is controlled, fluid is only temporizing.

In most trauma cases, the ideal resuscitation solution is blood. Prehospital blood product resuscitation administered to casualties in Afghanistan resulted in reduced mortality (12.2 vs. 18.2 %) for patients with moderate ISS (16–50) (Morrison, Oh et al. 2013). Currently, Israeli and British armed forces currently use blood products in the prehospital arena and there are ongoing trials with prehospital blood products on helicopter transports in US urban environments(Barkana, Stein et al. 1999; McLeod, Hodgetts et al. 2007). However, logistical problems related to storage have limited prehospital blood products use in most civilian and military US populations.

In the absence of blood, the ideal resuscitation fluid would be universally compatible, maintain circulating blood volume, minimize and/or correct coagulopathy, and deliver sufficient $O_2$ to tissues to prevent lethal sequelae caused by ATP depletion (Blackbourne, Czarnik et al. 2010).

Maintenance of intravascular volume can be achieved with crystalloids or colloids; however these fluids dilute clotting factors and platelets, which exacerbates coagulopathy (Barak, Rudin et al. 2004; Alam, Bice et al. 2009). The combination of coagulopathy and increased intravascular volume can increase the likelihood of clot disruption and ongoing bleeding (Sondeen, Coppes et al. 2003), and has been clinically confirmed (Bickell, Wall et al. 1994) Furthermore, asanguinous crystalloids or colloids are capable of carrying only about 0.3 ml of $O_2$ per 100 ml, which is not sufficient to sustain aerobic production of ATP. Many combat casualties resuscitated in the field with crystalloid or colloid alone arrive at the field hospital with acidosis (pH <7.25 and base deficit <6 mEq/L), hypothermia <36 °C, coagulopathy (international normalized ratio >1.5), tachycardia (heart rate > 105 beats/min), hypotension (systolic blood pressure (SBP) <110 mm Hg), and hematocrit <32 % and require massive transfusions (Schreiber, Perkins et al. 2007; Cancio, Wade et al. Cancio 2008; McLaughlin, Niles et al. 2008; Niles, McLaughlin et al. 2008).

## 7.4 Current Recommendations for Prehospital Resuscitation

In military populations, the first goal is to stop life-threatening external hemorrhage with manual compression, tourniquet, or Combat Gauze (QuikClot®, Connecticut USA). Tranexamic acid (TXA), an antifibrinolytic agent, should be considered if a casualty is anticipated to need significant blood transfusion CRASH-2 was a large randomized, placebo-controlled trial with over 20,000 trauma patients that demonstrated TXA reduced mortality (14.5 Vs. 16.0 %) and the risk of death due to bleeding (Shakur, Roberts et al. 2010). A similar reduction in mortality was demonstrated in military causalities who received TXA (Morrison, Dubose et al. 2012).Current guidelines instruct medics to give fluid only if the patient has an absent/weak radial pulse or altered mental status (in the absence of head injury) (McSwain, Salome et al. 2010; McSwain, Champion et al. 2011). Fluid resuscitation should be performed with 500 mL of 6 % hetastarch in lactated electrolyte buffer (Hextend®, Hospira Inc.), which can be repeated once if shock persists (CoTCCC 2011). 6 % hetastarch is recommended as the initial fluid of choice because of safety (Ogilvie, Pereira et al. 2010) and low volume/weight ratio compared to lactated Ringer's solution (Dubick 2011; McSwain, Champion et al. 2011).

Hypotensive resuscitation was first recognized as a successful strategy for combat casualty care in World Wars I and II (Cannon 1918; Beecher 1945), and was integrated into far-forward strategy in 1998 (Butler, Hagmann et al. 2000). If blood pressure monitoring is available the guidelines recommend targeting a SBP of 80–90 mm Hg (CoTCCC 2011). Hypotensive resuscitation is supported by

numerous studies (Owens, Watson et al. 1995; Hambly and Dutton 1996; Shoemaker, Peitzman et al. 1996). However in 1994, Bickell et al. performed the only prospective randomized trial on prehospital fluid resuscitation in hypotensive patients with penetrating torso injuries who were randomized to standard intravenous fluid or no fluid resuscitation. Estimated blood loss during emergency surgery, length of stay, and mortality rate were lower in those who received no prehospital fluid resuscitation (Bickell, Wall et al. 1994). Thus, there is a major unmet need for an HBOC or other improved resuscitation fluid if evacuation from the battlefield is likely to be prolonged, but only after the bleeding is stopped.

In civilians, there is a lack of evidence supporting the efficacy of even the most basic prehospital care by even the most highly trained civilian paramedics. In Los Angeles, private transportation was equivalent to emergency medicine service transportation in terms of outcomes at one level 1 center (Demetriades, Chan et al. 1996; Cornwell, Belzberg et al. 2000). In a Canadian study, no difference was found in outcomes between patients transported with basic life support versus advanced life support providers (Liberman, Mulder et al. 2003). Numerous studies have shown that minimizing prehospital resuscitation fluid improves outcomes (Bickell, Wall et al. 1994; Dutton, Mackenzie et al. 2002; Dretzke, Sandercock et al. 2004). This is summarized as the "scoop and run" being superior to the "stay and play" method.

An intervention as basic as the placement of prehospital vascular access is not supported by data, as it delays transport to a trauma center and no clear benefit has been identified (Pons, Moore et al. 1988; Honigman, Rohweder et al. 1990; Minville, Pianezza et al. 2006). Some studies have actually shown increases in mortality when intravenous access is placed by paramedics in the field (Smith, Bodai et al. 1985; Slovis, Herr et al. 1990; Cayten, Murphy et al. 1993). There is no consensus guidelines for all US prehospital providers, however in 2009 the EAST Practice Parameter Workgroup for Prehospital Fluid Resuscitation recommended that the placement of vascular access at the scene not be performed (Cotton, Jerome et al. 2009).

The EAST guidelines also state that for uncontrolled hemorrhage, aggressive prehospital fluid resuscitation is not beneficial and in some cases increases mortality (Kaweski, Sise et al. 1990; Bickell, Wall et al. 1994; Cotton, Guy et al. 2006). It is now clear that resuscitation should be withheld until active bleeding is controlled. If resuscitation fluid is administered in the prehospital environment it should be titrated for a palpable radial pulse using small fluid boluses. There is no consensus on the particular type of resuscitation fluid. If there is no clear evidence that even starting an IV has benefits in an urban trauma environment, due to the short transport time, it is not clear whether any added benefit could be demonstrated for an HBOC or other improved resuscitation fluid. On the other hand, in rural environments, prehospital transport times are likely to be prolonged, and an HBOC could prove beneficial.

There are several important differences between civilian and military populations. First, transport time can be much longer in military environments, especially

where ongoing combat does not allow for immediate casualty evacuation. Additionally, military providers have a tendency to follow evidence based guidelines more closely than their civilian counterparts, at least in the management of traumatic brain injuries, which improves outcomes (DuBose, Barmparas et al. 2011; Trunkey 2011).

## 7.5 Damage Control Resuscitation in a Hospital

In a hospital setting, resuscitation is best achieved by using PRBCs, plasma, and platelets in a 1:1:1 ratio. This concept is known as damage control resuscitation and has proven effective in both military and civilian populations (Holcomb 2003; Duchesne, Islam et al. 2009; Snyder, Weinberg et al. 2009). Plasma based resuscitation strategies (1 PRBC: 1 plasma) result in decreased mortality rates and improved correction of coagulopathy (Ketchum, Hess et al. 2006; Malone, Hess et al. 2006). Holcomb et al. performed a multicenter retrospective review of 22 centers and found that increased ratio of platelets to PRBC (1:1) improved early and late survival, decreased death due to hemorrhage and decreased multiple organ failure mortality rates (Holcomb, Zarzabal et al. 2011). Another component of damage control resuscitation is hypotensive resuscitation with a target SBP of 90 mm Hg (Bickell, Wall et al. 1994; Dutton, Mackenzie et al. 2002; Rhee, Koustova et al. 2003). Once the civilian or military trauma victim has arrived at a point of definitive care, it is unlikely that even an ideal HBOC would have any advantages over the unlimited blood products that would normally be available.

## 7.6 Advancing the State of the Art

Prehospital resuscitation is an area of trauma care with the greatest potential for improving outcomes and the area where an HBOC is likely to have its greatest benefit. This is underscored by the number of preventable deaths that occur in the prehospital environment in both civilian and military populations. Additionally, the prehospital resuscitation methods currently in use are essentially the same as technology available in 1831.

In order to advance the state of the art of fluid resuscitation, animal studies are necessary to evaluate new resuscitation fluids. It is important that these injuries closely resemble those in clinical trauma patients and that the treatment methods are relevant to the real life situation. For example, a constant pressure hemorrhage model (Wiggers Model) has been widely used to investigate hemorrhagic shock, but unfortunately does not replicate how trauma patients bleed. As COL Bellamy from Letterman Army Institute of Research stated "…No useful purpose is served by developing therapeutic interventions that are applicable only in nonexistent patient populations. The history of laboratory hemorrhagic shock research may be

a case in point because although many interventions have been proposed on the basis of animal experimentation, few if any have found a place in the treatment of human beings. For a laboratory shock model to have clinical relevance, it must replicate important aspects of shock as seen in human beings during or following massive blood loss…" (Bellamy, Maningas et al. 1986). The successful development of HBOC or any resuscitation fluid will depend on the use of clinically relevant animal models, which will provide the greatest opportunity for the result to translate into clinical success.

Presently, HBOCs are not in clinical use. Civilian and military trauma care experts met in 2010 at The Prehospital Fluid Conference sponsored by the US Army Institute of Surgical Research. The consensus at the time was that "…hemoglobin substitutes and perfluorocarbons are not ready for serious consideration…" (McSwain, Champion et al. 2011). However, the panel members were more hopeful about freeze dried plasma, and believe it has the greatest potential for improving resuscitation in the near future. Freeze dried plasma (FDP) was first used in World War II, but was terminated due to high rates of hepatitis transmission (Kendrick 1966). Recent, animal studies are promising (Shuja, Shults et al. 2008; Spoerke, Zink et al. 2009) and show that FDP can expand the blood volume while supplementing coagulation factors lost during hemorrhage (Hamilton, Van et al. 2011; Shuja, Finkelstein et al. 2011). In military casualties in Afghanistan treated at French military facilities, FDP has been shown to prevent and correct coagulopathy (Martinaud, Ausset et al. 2011). The US military is currently developing FDP products that are stable at ambient temperatures and has partnered with the French military under an expanded access investigational new drug protocol to use French-made FDP in far-forward operations (Blackbourne, Baer et al. 2012).

Despite the promising preclinical and early clinical results with FDP, a moment of pause should be observed. The history of HBOCs reveals numerous exciting and successful preclinical trials, but unfortunately these results did not translate into successful clinical trials.

The current endpoint of prehospital resuscitation in both civilian and military populations is the presence of a radial pulse. This is another example of how improvements can be made in the prehospital environment. There is currently no consensus on the endpoints of successful resuscitation, and clinical trials should be undertaken to identify clinically useful endpoints.

## 7.7 Summary and Conclusion

The failure of DCL-Hb and Poly-SFH-P clinical trials and the impracticality of prehospital blood product resuscitation, unfortunately restricts medical providers to resuscitate only with crystalloid, colloid, or no fluid. This underscores a major deficiency in prehospital medical care, for patients in severe hemorrhagic shock with no immediate access to a trauma center. An ideal fluid would expand blood

volume, maintain organ perfusion, prevent physiologic derangement, and replace coagulation proteins lost in hemorrhage.

Currently, prehospital resuscitation is performed with 6 % hetastarch for military providers (due to size limitations), and crystalloid or colloid for civilian providers. In both settings, hypotensive resuscitation is recommended and fluid should only be provided if SBP <90, absent or weak radial pulse, or altered mental status. Fluids should be limited until hemorrhage is controlled.

Effective prehospital resuscitation fluids are needed and further research will determine if HBOCs, FDP, or another agent will provide a survival benefit to severely injured trauma patients.

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# Part III
# Current Issues of HBOCs and Regulatory Framework

# Chapter 8
# NIH/FDA/DOD Interagency Working Group on Oxygen Therapeutics

**Phyllis Mitchell, Richard Weiskopf, Warren M. Zapol and Oxygen Therapeutics Working Group**

## 8.1 Introduction

Research and development in the field of oxygen therapeutics has made substantial progress in the past two decades. However, significant challenges have been encountered, as highlighted at an NIH/FDA/HHS workshop in 2008 (Silverman and Weiskopf 2009a, 2009b). The combination of challenges, negative perceptions, and current difficulties in obtaining adequate funding for continued research and product evaluation led the NIH/FDA/DOD to convene a working group of experts to examine the future of oxygen therapeutics in Boston in July 2011. The group examined the medical needs for oxygen therapeutics and outlined the basic and applied research needed to continue drug development for these clinical applications. This document summarizes the opinions of the working group.

A full list of the Working Group members are provided at the end of the article.

Source: National Heart, Lung, and Blood Institute; National Institutes of Health; U.S. Department of Health and Human Services (http://www.nhlbi.nih.gov/meetings/workshops/therapeutics.htm)

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## 8.2 Need

The Working Group's consensus is that there is a substantial unmet medical need for oxygen therapeutics and that lives could be saved if safe products were available. Oxygen carriers would be particularly valuable for patients in one or more of the categories discussed below: (a) "bridge to transfusion"; (b) alternative to red blood cell transfusion; (c) novel opportunities where red blood cell transfusion is not an established therapy.

### *8.2.1 "Bridge to Red Blood Cell Transfusion"*

This is an area where red blood cell transfusion is indicated and will remain necessary, but compatible red blood cells are not available in a timely manner. Examples are: (a) pre-hospital use in remote areas; (b) military in-theater use with long evacuation times, or complete unavailability of red cells; (c) patients who are difficult to cross-match and require red cells before a satisfactory cross match can be established and compatible units identified; (d) patients with rare blood types awaiting arrival of a compatible unit available from a distant location; (e) mass casualties.

### *8.2.2 Alternative to Red Blood Cell Transfusion*

This category is identified for use similar to red cell use, but when red cells cannot be used or when an oxygen therapeutic could be more satisfactory: (a) patient refusal to accept red cell transfusion (e.g. religious objection); (b) massive transfusion, to conserve the stored red cell resource, transfusing red cells only after hemostasis has been established; (c) treating anemic patients, who are transfused repetitively over a substantial period of time, in order to diminish immunological sensitization (e.g. sickle cell disease, thalassemia); (d) developing countries where red cell availability for transfusion is exceedingly limited or not available. In these regions no blood banking system may exist or the blood bank may have poor quality control (e.g. high viral infection rate).

### *8.2.3 Novel Opportunities Where Red Cell Transfusion is not an Established Therapy*

1. Treatment of acute ischemia when red cell transfusion would not be used ordinarily, including (a) myocardial ischemia due to arterial insufficiency; (b) cerebral ischemia due to major cerebral artery insufficiency; (c) acute limb

ischemia due to arterial insufficiency with reduced blood flow; (d) traumatic brain injury (e) sickle cell crisis, to prevent or treat areas of limited blood flow; (f) spinal cord ischemia; (g) reconstructive surgery- free or attached flaps.
2. Other novel opportunities include, for example: (a) carbon monoxide poisoning; (b) organ preservation (transplantation, cardiac surgery, etc.); (c) a component of a multi-functional resuscitation fluid.

## 8.3 Animal Models of Toxicity

Although current generation HBOCs were tested in a large number of well-defined animal studies, including models of hemorrhagic shock and anemia in normal animals, clinical trials with these products have been noted to have side effects that were not predicted by pre-clinical tests (Silverman and Weiskopf 2009, 2010). Examples of these serious adverse effects included excess morbidity, cardiac mortality, and pancreatitis, which were often associated with pre-existing conditions (Silverman and Weiskopf 2009, 2010). Consequently, the interagency working group strongly recommends that better animal models of various human disease states are needed to evaluate potential toxicity of oxygen therapeutics either as individual molecules or as a class of compounds (it should not be assumed that a particular side effect is associated with the entire class of compounds). The group believes that in the future, both as individual drugs, and classes of drugs, oxygen therapeutics should be evaluated in the presence of common clinical conditions. The working group believes two important and common disease pathways, endothelial dysfunction and oxidative stress, may have contributed to serious adverse events in prior clinical HBOC trials.

### *8.3.1 Models of Endothelial Dysfunction*

It has been known for decades that free hemoglobin avidly scavenges nitric oxide (NO). In the late 1990s, the HBOC field recognized that dioxygenation of NO by oxyhemoglobin was the major underlying cause of the rapid increase in blood pressure in animals and patients after administration of acellular hemoglobin. Major efforts were made to reduce the rate of this reaction by site-directed mutagenesis (Doherty et al. 1998) or by reducing in vivo extravasation by increasing HBOC molecular weight (Lieberthal et al. 1999; Knudson et al. 2003; Gould and Moss 1996; Nelson et al. 1992). It became clear that the vasoconstrictor and hypertensive effects of acellular hemoglobins and HBOCs, (Silverman and Weiskopf 2009, 2010) and gastro-intestinal symptoms in conscious humans (Viele et al. 1997) are due to scavenging NO, and that these side effects were potentiated

in animals with endothelial dysfunction. Possible dysfunction models include mice fed a high fat diet for 6 weeks, (Yu et al. 2010) diabetic mice (db/db), (Yu et al. 2010) or lambs with partial inhibition of NO synthesis by chemical blockade (Baron et al. 2012). In addition to regulating vascular tone, excess plasma hemoglobin may produce inflammation and platelet activation (Villagra et al. 2007; Boretti et al. 2009). Thus, the working group strongly advocates that HBOCs be studied in animal models of endothelial dysfunction to search for pulmonary and systemic vasoconstriction, and alterations of coagulation (including platelet activation), and inflammation. Coronary occlusion and pro-coagulant models, some of which have been studied for the effects of NO supplementation, could also be used to test the safety (also an area of potential therapeutic efficacy) of these molecules in diseased states (Schmidt et al. 2001). Effects of oxygen therapeutics on other pathways influencing endothelial-blood interactions, vascular reactivity and inflammation/platelet activation should be evaluated.

### *8.3.2 Models of Oxidative Stress*

Auto-oxidation of HBOCs causes the release of superoxide, which in turn rapidly dismutates to hydrogen peroxide and reacts with the newly formed methemoglobin to produce destructive protein-based radicals. All of these reactive oxygen species (ROS) are capable of producing tissue injury through lipid oxidation and protein degradation. This oxidative stress may be compounded by vasoconstriction and stimulate inflammatory responses, including the release of additional endogenous oxidant molecules. Methemoglobin itself is relatively unstable and rapidly loses heme. Free heme in turn can generate more ROS and, if present in high amounts, leads to iron overload pathology. Therefore physiological models examining oxidant stress, especially in animals without sufficient capacity for the production of anti-oxidants should be examined. There are a number of such animal models, including those that lack the ability to produce reducing molecules, such as ascorbic acid (e.g., guinea pigs, unlike most rodents, lack the ability to synthesize ascorbate) (Buehler et al. 2007; Butt et al. 2011). Other models for study include knockout mice without ascorbate synthesis (Koike et al. 2010).

Perfluorocarbon-based oxygen carriers are molecularly distinct from HBOCs, and have different pharmacodynamic, pharmacokinetic, and biologic activities. Thus, they may need to be studied in different animal models. Perfluorocarbons have high solubility for many gases, including oxygen. Further work on the basic mechanisms of oxygen delivery and alterations of NO signaling with perfluorocarbons would be beneficial. Animal models should include those mimicking common human diseases that might be expected in patients receiving these novel molecules. Studies of the effects of perfluorocarbons on microvascular and endothelial cell biology should be included in their development plans. Potential therapeutic areas for PFCs that might not pertain to HBOCs owing to the physical

properties of PFCs include treatment of gas embolism and decompression sickness. Research should include efforts to understand the mechanism by which PFCs induce transient thrombocytopenia.

## 8.4 Developing New Hemoglobin-based Oxygen Transporting Materials

Safety concerns have been identified in preclinical and clinical testing of HBOCs (Silverman and Weiskopf 2009, 2010) suggesting that further development of HBOCs and/or improved formulations are needed. We anticipate that studies in the additional animal models described above will help to identify further improvements in HBOC engineering and/or improved formulations and additives.

### *8.4.1 Further Engineering of HBOCs*

The study group recommends further research to develop entities designed to:

1. Lower rates of nitric oxide scavenging via inhibition of the dioxygenation reaction.
2. Inhibit extravasation into blood vessel walls.
3. Suppress damaging oxidative reactions.
4. Reduce heme loss and unfolding of HBOC protein.
5. Eliminate complement activation in normal animals and animal models of common human diseases.
6. Enhance pharmacokinetic properties, including extending the intra-vascular half-life.
7. Improve shelf life at room temperature.
8. Reduce oncotic pressure at high hemoglobin concentrations.
9. Enhance the $O_2$-carrying capacity of individual Hb molecules.

### *8.4.2 Improved Formulations and Additives*

The committee recommends further research to define any potential benefit for HBOC prototypes that may include entities that

1. Enhance production of NO from exogenous or added nitrite by augmenting the anaerobic nitrite reductase activity of deoxyhemoglobin.
2. Restore endothelial NO with bioactive molecules that release free NO or generate bioactive NO metabolites.

3. Are loaded with CO to reduce oxidative cellular metabolism and induce vasodilation.
4. Utilize co-administration of anti-oxidants or haptoglobin (Boretti et al. 2009) to reduce or eliminate oxidative pathway activation and heme release.

## 8.5 Improved Understanding of the Effect of the Oxygen Therapeutics on Tissue $PO_2$

Optimal development and evaluation of novel oxygen therapeutics requires improved methods to measure in vivo the $PO_2$ in vital tissues and organs in both animal models and humans. Such advances would provide the parameter of most importance to the therapeutic goal of administering oxygen therapeutics.

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# Chapter 9
# Regulatory Framework for Hemoglobin-Based Oxygen Carrier Trials

**Basil Golding**

## 9.1 Introduction

Oxygen therapeutics (OT) consist of hemoglobin-based oxygen carriers (HBOCs) and fluorocarbons. They have been developed to deliver oxygen ($O_2$) to tissues, mainly for treatment of shock due to blood loss. These products are being developed in order to treat blood loss when red blood cells are not available, as may occur on the battlefield or in civilian life when trauma occurs.

Investigational HBOCs have been shown in clinical trials to be associated with serious adverse effects including stroke, myocardial infarction, renal failure and death, and none have been approved by the FDA (Chen et al. 2009). As of this writing, FDA has issued a draft guidance addressing the criteria for safety and efficacy evaluation of oxygen therapeutics as red blood cell substitutes, but FDA has not issued the final, guidance (Guidance for Industry 2004).

Drug development of such products is not different from other drug products and involves, manufacturing under current Good Manufacturing Practice (GMP) conditions, biochemical characterization, pre-clinical studies, and clinical studies (Guidance for Industry: 2008). The focus of this chapter is on clinical studies that manufacturers may wish to consider for new generation HBOCs, (Guidance for Industry 2004).

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

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## 9.2 General Approach to Clinical Studies

Clinical trials should be performed according to Good Clinical Practice Guidelines (Guideline for Good Clinical Practice 2002) and follow a certain order that should also be applied to HBOCs under the investigational new drug (IND) provisions. These include Phase 1 safety studies, Phase 2 exploratory, and Phase 3 pivotal trials (21CFR312.21) (Guideline for Good Clinical Practice 2002). One challenge with HBOCs is that they are often intended for subjects in shock who may not be able to give the informed consent required for a clinical trial. Clinical trials with exception from informed consent are subject to specific conditions (21CFR50.24) which are discussed below. Clinical development programs of HBOCs can be complex, particularly if they involve clinical studies in severely injured patients with an exception from informed consent. Therefore, it is strongly advised that sponsors approach the FDA early in the development process to discuss potential study designs, including choice of study population, and other relevant matters.

Manufacturers generally are asked by FDA to perform studies in healthy volunteers and surgical patients before considering studies in the trauma population. This was the approach adopted by several manufacturers in their drug development programs for HBOCs (Jahr et al. 2012).

Prior to conduct of any clinical studies for any drug or biological product, those products are: manufactured under GMP conditions (21CFR 210.2); characterized in terms of purity and sterility; and tested in animals to assess safety and proof of principle for efficacy. Hemoglobin based products are tested for $O_2$ affinity ($P_{50}$), cooperativity (Hill co-efficient), and stability (Guidance for Industry 2004; Buehler and Alayash 2008).

An important safety concern for products derived from human or animal blood or tissues relates to preventing transmission of infectious agents. Biologics derived from human or animal tissue are subjected to multi-tiered safety measures including avoidance of contaminated sources, and viral clearance steps (i.e. inactivation and removal). Since HBOCs are derived from human or animal red blood cells or recombinant sources, the latter concern is pertinent. Human donors are screened for risk factors for infectious agents by questionnaires and their blood is tested for certain infectious agents (http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/BloodSafety/ucm095522.htm). Human plasma used to manufacture plasma derivatives is tested by nucleic acid tests (NAT) for several infectious agents, generally on pooled donor specimens. Viral clearance steps are required in the manufacturing of plasma derivatives including HBOCs. Animals are vaccinated against viruses and animal husbandry is optimized to reduce contact with infected animals (http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/ Guidances/UCM213415.pdf) (ICH Topic 2000). Additionally, any human and bovine source material may involve the risk of transmissible spongiform encephalopathy (TSE) (WHO 2006). Animal selection and other steps are taken to assure that the animals used are healthy and that the source material is removed in a facility that avoids possible cross-contamination

with TSE infectious agents (WHO 2006; Guidance for Industry 2010). Once a judgment can be made that a product is reasonably safe, based on its characterization and non-clinical data, it may be given to humans (21CFR312).

HBOCs have been shown to interfere with many standard clinical laboratory assays used in monitoring for patient safety. In vitro testing to determine to what extent a "correction factor" is needed, to account for this interference is usually completed before Phase 1 trials can begin (Kazmierczak et al. 1998).

Previous trials with HBOCs revealed serious adverse events in patients undergoing surgery or bleeding as a result of trauma (Guideline for Good Clinical Practice 2002). These have included stroke, myocardial infarction and death. These events were hypothesized as being due to binding to NO and causing ischemia due to vasoconstriction, and to endothelial damage due to release of heme and reactive oxygen substances (Buehler and Alayash 2008). These observations may influence the development of new HBOCs and may need to be considered in future human trials with HBOCs. For example, in order to reduce toxicity, a sponsor may seek to modify a new HBOC product or to co-administer another agent to demonstrate improved safety. Pre-clinical studies are typically performed in accordance with ICH S6 guidance (ICH 1997). Animals studies are important to evaluate a dose range so that "no observable adverse event levels (NOAELs)" can be defined (ICH 1997; ICH Topic E 8 1998). The NOAELs can then be utilized to determine a reasonable margin of safety for the initial dose in humans [21CFR312.23(a)(8)(ii)098].

## 9.3 Phase 1 Studies

Protocols for phase 1 studies [21CFR312.23(a)(6)(i)] are typically designed to establish the safety profile of the product in healthy volunteers (Guidance for Industry 2008; Guideline for Good Clinical Practice 2002). In the past, manufacturers have conducted studies that included a control group receiving an approved crystalloid or colloid. (Guidance for Industry 2008; Guideline for Good Clinical Practice 2002) FDA regulations require that subjects in a clinical trial should not be exposed to a product unless adequate safety information is available from non-clinical studies [21CFR312.23(a)(8)(ii)] and provided that unreasonable risk of illness or injury can be ruled out [21CFR312.42(b)(1)(iv), (2)(i)]. This is particularly applicable to healthy volunteers that do not stand to benefit from the trial. If the trial design does not meet FDA requirements, FDA can place the clinical trial on hold [21CFR 312.42(b)(2)(ii)].

Special attention to monitoring of adverse events of the nature seen in previous trials of HBOCs, would be appropriate. In the past trials, there were concerns with failure of multiple organ systems including heart (e.g. EKG, troponin) kidneys (e.g. creatinine and GFR), brain (e.g. neurological function), lungs (e.g. $pO_2$), and gastrointestinal (e.g. amylase and GI distress) (Guideline for Good Clinical Practice 2002; ICH Topic E 8 1998; Buehler et al. 2010).

In most studies investigators proceed with caution with a gradual dose escalation, i.e. starting with low doses and slowly increasing doses to define safe doses for later stage trials (Guideline for Good Clinical Practice 2002; Jahr et al. 2012). There are two approaches to dosing with large volume pharmaceutical like HBOCs, i.e. top-load (i.e. addition to existing blood volume) or exchange transfusion (removing blood while adding HBOC). For indications where large volumes of HBOC will be administered clinically, exchange transfusion approaches to dosing may be considered appropriate. The colloid osmotic effect and viscosity of certain HBOC solutions may add to the concern of fluid overload with these products in hemodiluted subjects, thus hemodynamic parameters should be monitored to avoid fluid overload (Xavier Monnet and Jean-Louis Teboul 2010).

The pace of enrollment will also depend on safety concerns. One subject at a time or several could be enrolled and observed before the next subject or group is exposed to the next highest dose. Again, depending on safety concerns, stopping rules may be required to avoid exposing additional individuals if the product is associated with serious adverse events (Guideline for Good Clinical Practice 2002; ICH 1997).

If the outcome of the phase 1 studies is satisfactory, i.e. no serious adverse reactions are observed, then phase 2 trials can proceed. Alternatively, if the benefit: risk calculation is favorable despite adverse events, then the product may be studied further (Guideline for Good Clinical Practice 2002; ICH 1997).

## 9.4 Phase 2 Studies

In general, phase 2 trials [21CFR312.23(6)(ii)] are performed on the target population to explore dosage, endpoints, and to obtain additional safety data.

With HBOCs evaluation, this is complicated by the fact that the target population usually identified is severe bleeding during trauma. Such subjects are often unable to provide informed consent for participation in a clinical study because they are in a life-threatening situation necessitating prompt medical intervention and time is insufficient to obtain the consent from legally authorized representatives (LAR). Recognizing the need to permit the study of safety and effectiveness of potential treatments for life-threatening emergencies to improve patient outcomes, FDA issued regulations that allow for a narrow exception from informed consent requirements for emergency research under 21 CFR 50.24. Trials performed with exception to informed consent raise additional regulatory issues that need to be addressed, including: (i) evidence that the product has a potential for direct benefit to study subjects and that current treatments are unsatisfactory or unproven; (ii) adequate public disclosure and community consultation; (iii) appropriate procedural steps to ensure that subjects' family members or LAR are informed of research enrollment; (iv) inability to identify prospectively individuals likely to become eligible to participate, and (v) collection of valid scientific evidence is necessary to determine the safety and effectiveness of the intervention.

A number of Phase 2 trials for HBOCs intended for severe bleeding in trauma were actually performed in surgical patients (Guideline for Good Clinical Practice 2002), partly because 21 CFR 50.24 regulations do not allow use of the exception from informed consent provisions if the clinical study could practicably be carried out without invoking the exception for emergency research. In previous trials, there were also difficulties associated with conducting the trials with the exception from informed consent (Guideline for Good Clinical Practice 2002). The outcomes were compared to patients receiving blood (Guideline for Good Clinical Practice 2002).

There are advantages to clinical trials with surgical rather than trauma patients. Elective surgery allows for a controlled environment with more stable patients, so that subjects can be carefully monitored. Attribution of adverse effects to the product is more easily assessed than in situations with unstable patients. Elective surgery allows patients to provide informed consent (discussed below) unlike trials in trauma patients where exception from informed consent is a complicating factor. In the past, clinical trials in surgical patients have been designed as randomized trials with HBOC in the test arm and red blood cells as the control arm (Guideline for Good Clinical Practice 2002; Jahr et al. 2012). The primary endpoint was mortality. Secondary endpoints that have been used include morbidity, avoidance of transfusions, and length of hospital stay (Guidance for Industry 2008; Guideline for Good Clinical Practice 2002). The primary endpoint of mortality has the advantage of being most objective and definitive (Guidance for Industry 2008). Statistical plans in the past have included a non-inferiority design comparing HBOCs to red blood cells with mortality as the primary endpoint (Jahr et al. 2012). The implicit objective of such studies was to demonstrate indirectly the superiority of trauma resuscitation with an HBOC compared with colloid or crystalloid, while recognizing the infeasibility to randomize patients to an asanguinous control when blood products are available.

Phase 2 trials are exploratory in nature and may provide sufficient information to allow for the design of pivotal trials. The objectives of Phase 2 trials include: (i) determining optimal dosing by investigating different doses with different rates of delivery; (ii) establishing which primary and secondary endpoints to use in the pivotal trial; and (iii) expanding the safety database in different patient (e.g. different surgery indicated) groups [21CFR312.23(ii)](11, 12).

If satisfactory results are obtained in Phase 2 surgical trials, the next step could be a Phase 2 trial in trauma patients (ICH 1997; ICH Topic E 8 1998). The value of a Phase 2 trial in trauma before embarking on a pivotal trial, would be not only to further determine dosage, establish endpoints, and investigate the safety database in the target population, but also to explore the criteria for identifying subjects that may benefit from the treatment. This could involve use of a scoring system to determine inclusion and exclusion criteria (Yücel et al. 2006).

A Phase 2 trauma trial could be conducted in the ER and thus subjects could be assessed and monitored under more controlled conditions than in a pre-hospital setting. The design may be similar to the surgical trials, in that red blood cells would be the comparator. Ethicists have questioned the appropriateness of a trial in

which use of an HBOC in comparison to colloid or crystalloid was permitted to continue in the ER when blood was available (Chen et al. 2009).

The risks of HBOC administration must be offset by potential benefits. This is where inclusion and exclusion criteria become important. Knowing the risks of dying from trauma for a particular individual, based on a scoring system and supporting database, could greatly facilitate making a reasonable predictive benefit: risk calculation. An example of such a scoring system is the TASH score (Yücel et al. 2006).; whereas the National Trauma Database can be accessed to obtain outcome information (Meredith et al. 2003).

Phase 2 trials are not necessarily powered to show efficacy with statistical significance, but sample sizes should be sufficient for the safety and efficacy data to provide the basis for deciding whether to proceed with a large pivotal trial. If the data show a favorable trend in efficacy and safety supporting a conclusion that there is a reasonable prospect of direct benefit to study subjects, then the sponsor may seek to perform a phase 3 trial, possibly under the exception from informed consent provisions for emergency research as stated in 21CFR50.24.

## 9.5 Phase 3 Studies

Once Phase 2 trials have shown sufficient safety and efficacy data, it may be reasonable to consider a pivotal Phase 3 trial in trauma. Previously these trials were designed as multicenter randomized controlled trials, powered at 80 % or higher to show superiority over crystalloid, with mortality as the primary endpoint (Guideline for Good Clinical Practice 2002). Blinding of the health care providers to the identity of the HBOC or control fluid may not be possible because of the distinctive color of the HBOC, but those involved in analyzing the data can be blinded.

Prior trauma trials with HBOCs have included an independent data monitoring board (DMB) with well-defined stopping rules and a statistical plan to include interim analyses for safety and futility. A DMB is required for trials performed with exception to informed consent (Guidance for Institutional Review Boards 2011).

### *9.5.1 Benefit:Risk Calculation*

Benefit: Risk calculation plays an important role in decision-making to allow a clinical trial to proceed, but is especially relevant when exception from informed consent is involved (21CFR50.24).

In previous studies, the safety profiles from the Phase1/2 trials have formed a basis for making a benefit:risk assessment. The potential benefit was largely dependent on selection of the target population and knowledge of outcomes with

standard care. This was derived from the literature and databases. The known mortality rate for a particular group of trauma subjects, defined by a scoring system (Yücel et al. 2006; Meredith et al. 2003), was estimated, and the potential reduction in mortality of the investigational HBOC based on animal and human studies (Guidance for Industry 2008; Guideline for Good Clinical Practice 2002) were used to arrive at a benefit:risk calculation to decide whether the trial had a reasonable potential to benefit the subjects.

Secondary efficacy endpoints such as morbidity, length of hospital stay, and the number of red blood cell units transfused, were included in previous trials (Guidance for Industry 2008; Guideline for Good Clinical Practice 2002).

In previous trials, safety was monitored carefully including use of an independent DMB to follow patient safety during progress of the trial (ICH 1997; ICH Topic E 8 1998; Guidance for Institutional Review Boards 2011). The DMB was convened at certain time-points during the trial to determine whether: (i) the trial should continue; (ii) be terminated for futility; or (iii) be terminated because of safety concerns. In addition, stopping rules were in place to stop the trial if adverse events occur at a higher rate than expected as defined a priori (ICH 1997; ICH Topic E 8 1998; Guidance for Institutional Review Boards 2011).

## 9.6 Conclusion

In conclusion, clinical trials of HBOCs have followed a conventional approach of a series of studies intended to minimize patient risks during product development. In particular, studies in well monitored surgical patients have been required before studies in trauma patients, which generally require exception from informed consent, could proceed. Because clinical development programs of HBOCs can be complex, particularly if they involve clinical studies with the exception from informed consent, FDA input should be sought early on to assure adequacy of the approach to clinical trials.

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# Part IV
# Approaches to HBOCs

# Chapter 10
# HBOCs from Chemical Modification of Hb

**Ronald Kluger and Francine E. Lui**

## 10.1 Fundamentals for Haemoglobin-Based Oxygen Carriers

We have produced several recent reviews related to the technology of chemical stabilization of haemoglobin and a brief view of the future of HBOCs (Kluger 2010; Lui and Kluger 2010). This chapter is intended to provide a broader perspective of the topic of chemical stabilization for the production of HBOCs.

### *10.1.1 Haemoglobin and Oxygen Binding*

Haemoglobins are the oxygen-carrying components of red cella in animals, providing a precisely controlled machine for the acquisition and distribution of atmospheric oxygen within an organism. While human adult haemoglobin (Hb A) is the focus of much of the interest in HBOCs, other sources such as bovine or porcine haemoglobin have been used. However, in this chapter the abbreviation Hb will specifically refer to HbA. Hb is a globular 64 kDa tetrameric assembly of globin subunits consisting of two identical $\alpha\beta$-dimers. Each monomeric subunit is associated with a ferrous heme to which oxygen binds reversibly. The quaternary structure of Hb is complex and there is significant variation between the three-dimensional structure of deoxy-Hb (T-state) and ligated oxy-Hb (R-state), with a 15° shift between the $\alpha\beta$ interfaces of the tetrameric protein. The ability of Hb to bind and release oxygen can be assessed at the level of detail needed to design an HBOC using two parameters: oxygen affinity (characterized by $P_{50}$), which measures the average energy of binding of the four oxygen molecules to a tetramer

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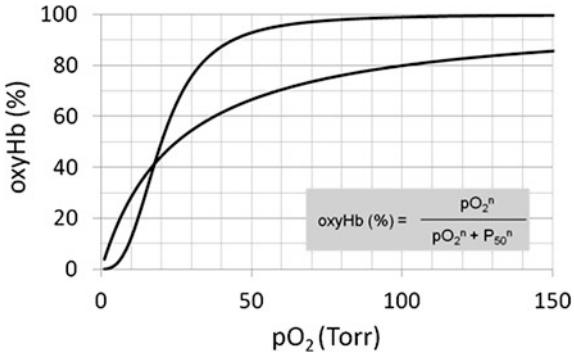


**Fig. 10.1** The oxygen dissociation curve of haemoglobin is governed by the oxygen dissociation equation. The sigmodial curve of haemoglobin ($n_{50} = 3$) indicates allosteric cooperativity, and allows for haemoglobin to be more $O_2$-saturated in the lungs at high oxygen tensions and more effectively deoxygenated at lower oxygen tensions such as in the capillaries. Myoglobin is an oxygen-carrying protein that does not display cooperativity—its oxygen affinity curve is hyperbolic ($n_{50} = 1$)

and the cooperativity of sequential oxygenation (characterized by the Hill coefficient, $n_{50}$), which measures the extent to which the oxygenation curve is sigmoidal (the result of cooperativity) versus hyperbolic (where there is no cooperativity). A comparative set of cooperative and hyperbolic oxygenation curves is shown in Fig. 10.1.

Oxygen affinity is evaluated from a plot that relates the extent of occupancy of oxygen-binding sites on Hb in solution to the partial pressure of oxygen ($P_{O2}$) in the environment of the measurement (Fig. 10.1). The inherent oxygen affinity of Hb is quite high and in circulation the tendency would be for the oxygen to be retained by Hb rather than being released in hypoxic environments. However, the affinity is significantly reduced by allosteric effectors in the red cell, including protons,(Perutz 1990) 2,3-disphosphoglycerate [2,3-DPG](Benesch and Benesch 1967), chloride and carbon dioxide. Acellular Hb that has been stripped of 2,3-DPG has a higher oxygen affinity ($P_{50} = 13.3$ torr) compared to Hb in red cells where 2,3-DPG is present at a high concentration, giving $P_{50} = 26$ torr (Riess 2001).

The cooperativity of binding of oxygen to Hb is conveniently measured by the Hill coefficient, where $n_{50} = 3$ for native Hb indicates the presence of substantial sigmoidal character in the oxygenation curve (Bellelli 2010). This positive cooperativity in oxygen binding is manifested as the increased affinity of the remaining set of binding sites for oxygen. A decrease in $n_{50}$ is indicative of decreased cooperativity. The cooperative oxygenation behavior of Hb achieves a highly controlled and effective delivery of oxygen within a small physiological oxygen tension range (100 torr in arterial blood and 30 torr in venous blood).

### 10.1.2 Interaction with Ligands Other than Oxygen

While the Fe(II) heme center of haemoglobin performs effectively in acquiring and delivering oxygen function, it also binds other diatomic molecules, most notably carbon monoxide and nitric oxide.

### 10.1.3 Carbon Monoxide

The association between Fe(II)-heme and CO is well-studied and the combination is reversible by introduction of visible light and nitrogen to produce deoxyhaemoglobin (Perrella 1999). CO is also released spontaneously but slowly and while it is present on the heme, it prevents addition of oxygen. The relatively stable bond between CO and Fe(II)-heme has some utility in preparation and administration of an HBOC. The stable bond between CO and Fe(II)-heme keeps the Hb in the ferrous state and eliminates problems from oxidation of the Fe(II)-heme iron to the non-functional Fe(III)-heme state that occurs in the presence of oxygen. (Vandegriff et al. 2008) HBOCs have been tested that are administered in the carbonmonoxy form (Sangart's MP4CO). The amount of CO that is present in one equivalent of the protein is so small that its release is not a problem with respect to the toxic effects of CO, which are associated with blocking the respiratory chain. In general, a CO-derived material is resistant to oxidation and CO will be released in circulation. The CO release may contribute to anti-inflammatory, anti-apoptotic and anti-proliferative effects that can be advantageous (Ryter and Otterbein 2004). The oxy-deoxy cycle of the material will be fully functional as the CO is released. In particular the benefits of utilizing CO-derivatives as HBOCs for clinical trials should have significant advantages in stabilizing the product before administration.

### 10.1.4 Nitric Oxide

Nitric oxide (NO) is an endogenous vasodilator that plays a critical role in smooth muscle relaxation (Bian and Murad 2003). NO binds to the same heme sites as does oxygen and it does so with a very high affinity. Once bound, it can oxidize the heme to produce metHb (Doherty et al. 1998). The process reduces the bioavailability of endothelium-derived NO and can lead to vasoconstriction and hypertension. Hb within red cells has limited interaction with NO because the unstirred layer surrounding the erythrocyte membrane forms a diffusional barrier between NO and Hb. Moreover, the intravascular laminar flow creates an RBC-free zone that consists only of plasma flowing along the endothelium (Liao et al. 1999). Outside the red cell, acellular Hb scavenges endothelial NO, primarily through its ability extravasate through the endothelium (Matheson et al. 2002;

Sampei et al. 2005; Cabrales et al. 2009). Had this been known early in the quest for an effective HBOC, designs that would avoid this problem would have been an essential feature of every approach, since many potential HBOCs were similar to extracellular Hb. Eventually, the observation of vasoactivity of potential HBOCs in clinical trials led to the ending of most trials (Natanson et al. 2008). The tight binding of NO to Fe(II) heme and its ability to oxidize the ferrous heme to the ferric state was seen as the key factor in understanding the vasoactivity of the HBOC candidates and the critical factor to be overcome in developing a safe and effective product. While oxygenation characteristics of any HBOC are certainly important for efficacy, interactions with NO can cause serious enough problems with safety that they must be overcome in any product. With respect to dealing with NO issues, the possibilities are (Kluger 2010) an Hb derivative that binds $O_2$ but not NO, (Lui and Kluger 2010) an Hb derivative that generates NO from other species in circulation (Perutz 1990), an Hb derivative that does not extravasate through the endothelium.

### *10.1.5 HBOCs from Chemical Modification of Haemoglobin*

Early attempts at converting Hb to an HBOC started as standard exercises in protein stabilization. These involved using established chemical cross-linking reagents to produce linkages between protein side chains that prevent dissociation of the tetramer (Keipert et al. 1982). It is thus instructive to review design criteria that were considered prior to extensive clinical testing. Ideally, the reagent that is used to convert Hb into an HBOC should introduce chemical linkages only where they would be likely to prevent dissociation of the functional tetrameric ($\alpha\beta\alpha\beta$) protein into its nonfunctional constituent $\alpha\beta$-dimers, without introducing additional modifications that make the result heterogeneous. Logically, the resulting candidate for HBOC status should have oxygen binding properties that meet theoretical requirements that are based on known physiological parameters for circulating red cells. Initially, it was logically assumed that this could simply be based on an analogy to the oxygenation properties of whole blood ($p_{50} = 26$). As well, it is desirable to have analytical methods that enable the sites at which the protein had been modified to be identified. This would relate chemical modifications to critical functional properties.

Where a material has suitable stability and oxygenation properties, production on a large scale is necessary for *in vivo* pre-clinical testing of toxicity, metabolism, safety, and efficacy. Where these tests give desirable results, the reactions must be developed to be run on a larger scale in order to provide sufficient material for clinical testing. In addition, dealing appropriately with NO binding has to be achieved.

## 10.2 The Need for Chemical Modification

### *10.2.1 Preservation of Tetrameric Form and Function*

As long as Hb is retained within the red cell it remains effective as an oxygen carrier. Outside the red cell native Hb is converted into its components by the liver. Its long-term effectiveness in circulation is due to its location *within* the red cell. The tetrameric form ($\alpha_2\beta_2$) is in equilibrium with its constituent $\alpha\beta$-dimers. The high concentration of the protein within the cell causes Hb to be maintained in the functional tetrameric state. As mentioned earlier, the presence of allosteric effectors within the cell also controls the extent of oxygenation. Most notably, 2,3-DPG enhances the ability of oxygenated Hb to release oxygen by preserving the low-affinity T-state structure of the protein (Benesch et al. 1972). In addition, enzymes (catalase and superoxide dismutase) within the cell catalyze the destruction of reactive oxygen species, such as peroxide and superoxide, that arise from the interaction of the ferrous heme and bound oxygen (D'Agnillo and Chang 1998). The inevitable auto-oxidation of the ferrous heme to the nonfunctional ferric state is reversed by metHb reductase (Huennekens et al. 1957).

In circulation outside a cell, Hb quickly becomes ineffective as an oxygen carrier for a variety of reasons: irreversible dissociation of the tetramer into $\alpha\beta$-dimers (Ackers and Halvorson 1974), oxidation to ferric metHb (metHb), and the absence of effectors. The lack of protection that is available from the cell membrane of red blood cells and the helper enzymes within causes the Hb-dimers not only to be ineffective as oxygen carriers, the smaller species eventually become a source of physiological stress and organ damage. The haemoglobin subunit dimers are taken up through circulation within the liver, forming a complex with haptoglobin (Ship et al. 2005; Chow et al. 2008; Boretti et al. 2009). The complex is processed in the hepatosomes where the components of the protein are prepared to be recycled. If large amounts of free Hb (tetramers or dimers) are present, the hepatosomes become fully occupied and the liver is bypassed. The dimers are then excreted through the glomerulus of the kidneys (Keipert et al. 1982; Feola et al. 1988). This leads to haemoglobinuria and renal injury. Thus, at the very least, a successful HBOC would have to retain the tetrameric state of Hb. This serves to minimized the release of dimers and the harmful effects in the kidney (Winslow 2006). This can be achieved either by encapsulating Hb into a lipid membrane (Chap. 11), or by chemical modifications to the protein that maintain the functional tetramer-like structure.

### *10.2.2 Alterations to Oxygen-Binding Properties*

Modifications that maintain Hb in a tetrameric form alter the physical properties of the protein as well as, the functional properties. The most obvious functional changes are in the oxygen binding properties ($P_{50}$ and $n_{50}$) of the modified protein.

Thus, each modified Hb binds and releases oxygen differently. These changes to oxygen binding are readily assessed by measurements of oxygenation curves of pure components against oxygen tension ($pO_2$) while stability is followed in the visible absorption peaks of oxy- and deoxy-heme proteins as well as CD spectra.

A higher value of $P_{50}$ for the modified protein compared to unmodified Hb indicates that the modification lowers the energy of binding to oxygen (by favoring the *T*-state to a greater extent), while a lowered $P_{50}$ indicates a higher oxygen affinity (favoring the *R*-state). We can think of these effects as being the result of cross-linking that creates points of resistance to conformational change necessary for cooperativity in oxygen binding. The effect on affinity ($P_{50}$) is due to the linker preventing relaxation of the chains to a lower energy R-state (Kluger et al. 1996), while reduced Hill coefficients ($n_{50}$), result from disruption of the overall environment of the solution around the protein. Since the designated function of an HBOC is oxygen delivery, the efficiency of delivery will be the key factor in determining how much material is needed to replace red cells in their functional capacity. As the established use of banked red cells is to replace lost red cells, it is logical to do a straight replacement of what has been lost. On the other hand, Hb within an HBOC can be more or less effective compared to the same amount of Hb within a red cell and the amount to be used is more appropriately based on the efficacy of oxygen transport of the material as determined by the oxygenation parameters.

## 10.3 Approaches to Hb Modification for Use as HBOCs

A common set of reagents used for modification of Hb in general includes electrophiles that react with nucleophilic functional groups on side chains of amino acids (Jones et al. 1993; Kluger 1994; Kluger et al. 1994; Wodzinska and Kluger 2008) (Fig. 10.2). These side chain groups react with synthetically prepared electrophilic reagents that can be modified for specific modification of the protein.

### *10.3.1 Regiospecificity, Homogeneity/Heterogeneity*

A reagent that is specific for a particular functional group on the protein will react usually give multiple products because the functional group will be at more than one site on each subunit. Reaction with such a reagents produces a highly heterogeneous mixture of altered proteins. In contrast, a reagent can be designed to react with limited subsets of a reactive functional group based on differences in local environments. With this added specificity, certain cross-linked Hb derivatives will be formed preferentially, creating at outcome that is potentially homogeneous or one that contains very few components. Another complication of a less

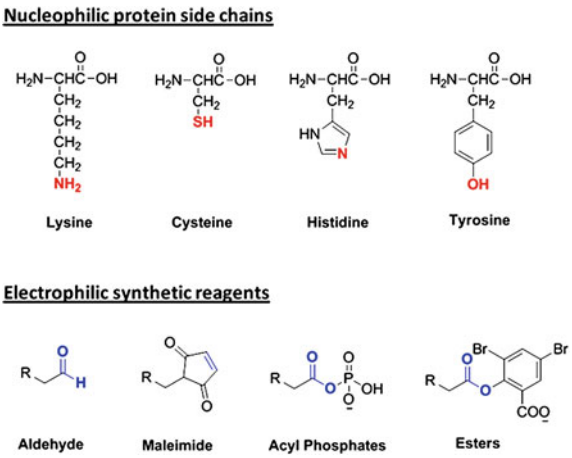


**Fig. 10.2** The nucleophilic site of protein side chains (in *red*) react with electrophilic synthetic reagents (in *blue*) to create stable covalent bonds. Electrophilic synthetic reagents can either be general reagents that react with most protein side chains, or they can be chemically modified to include certain groups that contribute to site specificity

specific reagent is that reaction can continue and lead to uncontrolled polymeric derivatives (see Fig. 10.3).

The source of regiospecificity in chemical reactions is normally the result of the inherent chemical properties of the functional group that reacts and the functionality of nearest neighbors. In contrast, regiospecificity of a reaction within a protein is the result of chemical properties within the regional environment of the potential reaction sites (Jones et al. 1993; Keipert and Chang 1988; Martinek and Torchilin 1988). In this sense, a reagent that is regiospecific within a protein is described as being "site-directed" or "site-selective". In dealing with cross-linking, these reagents are typically bi-functionally reactive (reactive groups on both ends). In that case, regiospecificity is essential because two different parts of the reagent are reacting. If one end is site-selective and the other is not, either end may react first, limiting the possibilities for the second step.

## *10.3.2 Intra- and Inter-Molecular Linking*

Cross-linking reagents are normally designed for *intra*-molecular linking (within Hb tetrameric subunits). Linking reagents are less common and are designed for *inter*-molecular linking (between multiple Hb tetramers). When Hb is modified selectively to give intra-molecular cross-linking the process requires a very selective reagent. The resulting chemically stabilized Hb tetramers ($\sim$64 kDa) are referred to as "cross-linked Hbs".

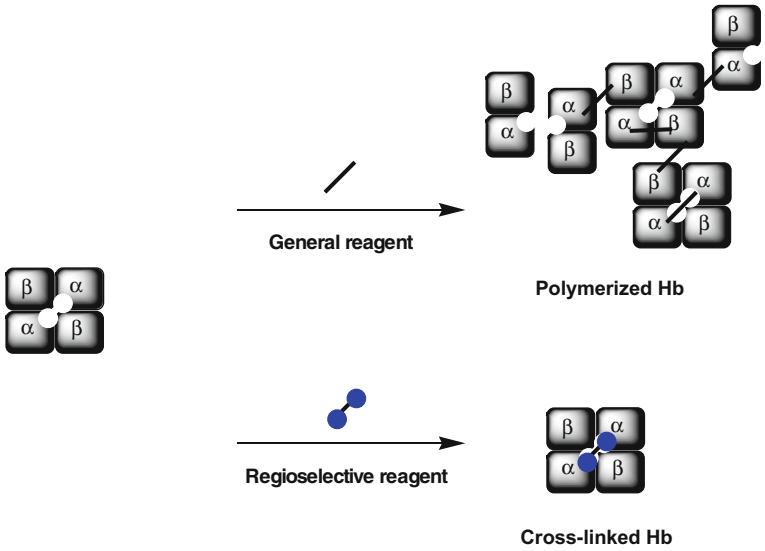


**Fig. 10.3** A general chemical reagent does not discriminate between sites and will react with any surface accessible residue to create non-specific cross-links. Many polymerized Hbs are produced with general reagents. A regioselective reagent has chemical groups (depicted in *blue*) that can direct the cross-linker to a specific site, such as through an electrostatic interaction

## 10.4 Intra-Molecular Cross-Linking

### *10.4.1 The DPG-Binding Site and Chemical Reagents*

One of the sites within Hb that has been targeted for site-selective reaction is the pocket within the Hb tetramer that binds the endogenous allosteric effector: 2,3-diphosphoglycerate (DPG) (Fig. 10.4). The negatively charged penta-anionic DPG is attracted electrostatically to the polycationic DPG-binding site on Hb, which contains multiple protonated amino groups derived from the side chains of lysine residues. The protons on the amino groups in the DPG-binding site are in dynamic equilibrium between the amino groups so that the amino groups are also available as reaction sites (Benesch and Benesch 1967; Benesch et al. 1972). Thus, the DPG-site-selectivity of anions can be used as the basis of an approach to making a cross-linking reagent site-specific. Since reagents commonly react as electrophiles, the cross-linking "war head" should be an anionic electrophile. Since anions are nucleophiles the two characteristics must be contained in adjacent functionalities. The electrostatic forces that direct DPG to its cationic binding site will then direct an anionic cross-linker to the DPG binding site. This ensures that the reagent reacts at a known site, rather than at multiple surface available sites.

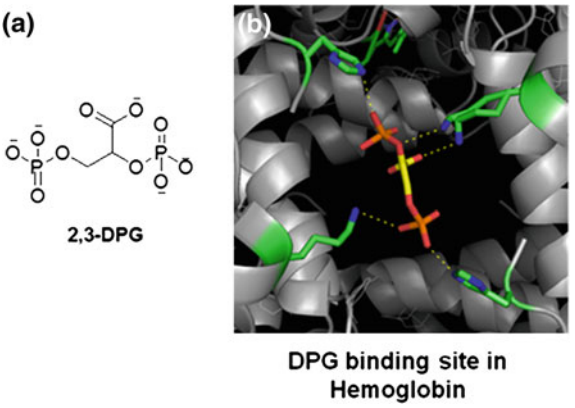


**Fig. 10.4** **a** The natural allosteric effector 2,3-diphosphoglycerate (2,3-DPG) is a penta-anionic molecule that binds to **b** the polycationic DPG-binding site within Hb (Figure obtained using PyMOL v1.5 and PDB code 1B86)

## *10.4.2 αα-Cross-Linked Hb (DCLHb)*

An early and impressive example of a site-specific modification being used to produce a pure cross-linked tetramer was based on observations by Klotz and coworkers. They observed that acetyl salicylic acid (ASA, aspirin), which is an anion and an electrophile in neutral solution, reacts only with amino groups in the DPG binding site of sickle Hb, explaining the anti-sickling effects of that drug (Klotz and Tam 1973) (Fig. 10.5). Further evaluation revealed that this selectivity by anionic aspirin derivatives for the DPG site in all Hbs is best achieved by using 3,5-dibromosalicylate (DBS) esters. Klotz and Walder showed in particular that the bifunctional 3,5-dibromosalicylate ester derivative of fumaric acid (bis(3,5-dibromosalicyl) fumarate, DBSF), can be a highly efficient and selective cross-linker (Snyder et al. 1987; Walder et al. 1979).

As noted above, polyanions bind selectively to the polycationic site that binds DPG in the red cell (Benesch et al. 1972). With the site occupied by polyanions, anionic cross-linkers are unable to bind and therefore do not react in competition. They are still able to react at the other end of the site with high selectivity for the ε-amino group of each α-99 lysine. Thus, the only product from the reaction of deoxyHb with DBSF in the presence of DPG or IHP has a fumaryl cross-link

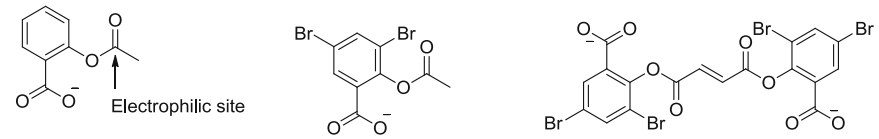


**Fig. 10.5** Aspirin and its dibromo (DBS) anionic derivatives target the cationic DPG binding site within the *β*-subunits. The bifunctional reagent bis (3,5-dibromosalicyl fumarate), DBSF creates specific cross-links based on the reactivity of salicylates with Hb

between the α-99 lysine side chains (Snyder et al. 1987). This cross-linked Hb is αα-fumaryl Hb. A product from Baxter that has this structure was give the trade name DCLHb, an acronym for "Diaspirin cross-linked Hb". The similar publicly disclosed species was called ααHb and was studied extensively by Winslow (Vandegriff et al. 1988; Winslow 1989; Keipert et al. 1994). The cross-linked tetrame has a molecular weight of about 64 kDa, with $P_{50} = 32$ mmHg and $n_{50} = 2.6$. DCLHB was the product evaluated as an HBOC by Baxter laboratories (Winslow et al. 1988; Estep et al. 2008).

In order to understand the specificity and reactivity of the DBS groups with Hb, a systematic evaluation of chemical derivatives of the 3,5-dibromosalicylate leaving group was carried out by De Stefano and Kluger (Kluger and De Stefano 2000). This was done to understand how DBSF reacts within the DPG-binding site. The correlation of reactivity against the basicity of the leaving group in the reaction with propylamine (a mimic of a protein side chain) showed that 3,5-dibromosalicylates are much more reactive than predicted from their $pK_A$ (approximately 15 times faster) based on other salicylates. In fact, in the reaction with Hb there is a further advantage in the reactions of 3,5-dibromosubstituted esters compared to the unhalogenated analogues. The yield of specifically modified Hb and the minimal level of hydrolysis make them superior to other derivatives that were tested. Examination of the structure of the reagents suggests that it is likely that the size and orientation of the bromine atoms provide a large steric bulk that directs the reaction to an amino group near the surface of the protein at the highly cationic DPG binding site. In addition, the adjacent carboxyl on the leaving group can also assist in promoting the nucleophilic amino group of lysine-82 of the *β*-subunit to become deprotonated to a larger extent by changes to the local polarity.

### *10.4.3 Cross-Linked Tetramers and Vasoactivity*

Two explanations have been proposed for the toxic effects associated with administration of first generation HBOCs. One theory is that HBOCs elicit adverse effects, including vasoconstriction by scavenging nitric oxide (NO), the vasodilator produced by the endothelium. It had been established in the 1990s that the endothelial relaxation factor (ERF), the signal for vasodilation for relaxation of the muscle surrounding blood vessels is nitric oxide (NO) (Furchgott et al. 1992; Bian and Murad 2003). This shares metal-binding properties with oxygen and associates strongly with ferrous atoms within the hemes of Hb. A logical and consistent explanation of the problem is that the tested HBOCs, unlike red cells, are able to permeate the endothelium. The localized depletion of NO would be expected to block relaxation of blood vessels (Sampei et al. 2005). In a critical test of the possible sources of vasoactivity, Zapol and co-workers demonstrated that intravenous infusion of either murine tetrameric Hb or a well-studied HBOC induced prolonged systemic vasoconstriction in wild-type mice but not in mice congenitally deficient in endothelial nitric oxide synthase (NOS3) (Yu et al. 2008, 2009).

Mice that could not produce NO in their endothelium did not succumb to the vasoconstrictive effect of HBOCs. (Yu et al. 2008, 2010 Vaporidi et al. 2010).

Winslow and coworkers proposed that HBOCs with low oxygen affinity (high $P_{50}$) can release oxygen in systemic arterioles, an effect that can induce a vasoconstrictive homeostatic response that limits oxidative stress (Tsai et al. 2003). They concluded that an HBOC with high oxygen affinity (low $P_{50}$) will instead facilitate delivery of oxygen to sites in the capillaries with low oxygen levels. Originally, it was believed that an HBOC with oxygen affinity close to that of RBCs ($P_{50} = 28$ torr) will function effectively. However, it was proposed that the small size of the cross-linked Hb tetramer leads to a mode of oxygen transport that is different from that of RBCs, although the molecule carrying oxygen is essentially the same (Cole et al. 2008). The decreased red cell concentration near vessel walls result I a zone with no oxygen source and thus there is an increased distance for oxygen to diffuse to tissues from RBCs. In contrast, the small size of modified cell-free haemoglobin will allow oxygen to diffuse readily within the lumen, increasing lateral oxygen transport. This is the basis of "facilitated diffusion" that is mediated by small, highly diffusible HBOCs that increase lateral transport by acting as carrier proteins. Designs based on this hypothesis create HBOCs with higher oxygen affinity than red cells to avoid homeostatic responses and to promote movement of oxygen from RBCs to surrounding tissues. The higher affinity carrier attracts oxygen from the red cells and transports it to the endothelial area.

### *10.4.4 Acyl Phosphate Reagents*

The regioselectivity demonstrated by the anionic acylating DBS has been cited by Klotz as the key to the functioning of salicylate derivatives. It follows that other anionic reagents that are negatively charged will share the same specificity for the DPG binding site in Hb. Manning proposed that members of another class of anionic acylation agents, acyl phosphate monoesters, would also be effective antisickling agents (Ueno et al. 1985, 1986; Ueno and Manning 1988). This was based on an earlier report by Tsui and Kluger that methyl acetyl phosphate is an effective anionic acylation agent (Kluger and Tsui 1980, 1986).

Ueno and Manning showed that methyl acetyl phosphate selectively acylates the amino groups of the $\beta$ subunits within the DPG-binding site of HbA, making the specificity similar to that of the reaction of the 3,5-dibromosalicylates (Ueno et al. 1986) (Fig. 10.6). A method of producing a variety of cross-linkers based on acyl phosphate monoesters was developed from readily available bis-acid chlorides as precursors (Grant et al. 1988; Kluger et al. 1990). This permitted the construction of cross-linking derivatives with a defined site of reaction and defined cross-link spans by straightforward chemical reactions (Kluger et al. 1994, 1996). These reagents are efficient, water soluble, and react with a great degree of specificity with amino groups of the $\beta$-subunits in the DPG binding site. The acyl phosphate analogous to DBSF, FBMP, is shown below (Jones et al. 1993). FBMP

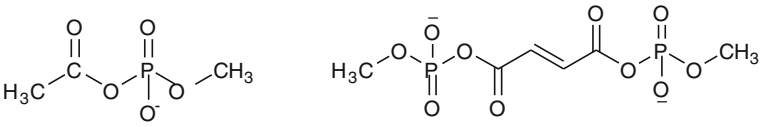


**Fig. 10.6** Acyl phosphate groups such as the simple methyl acyl phosphate are also anionic directing groups that can react within the DPG-binding site of HbA. Fumaryl bismethyl phosphate (FBMP) is ab alternative anionic electrophilic reagent that can cross-link residues in the DPG-binding site of Hb, stabilizing its tetrameric state

reacts with deoxygenated Hb to produce cross-links between an α-amino group of β-Val-1 and the ε-amino group of β-Lys-82 as well as between the two β-Lys-82 amino groups.

### *10.4.5 Effects of Cross-Link on Oxygen Affinity*

A surprising discovery was made during the evaluation of the oxygenation properties of the cross-linked products: the oxygen affinity of the resulting cross-linked protein, when the link is between the α-amino group of β-Val-1 and the ε-amino group of β-Lys-82, is directly related to the span of the cross-link. An inverse linear relationship between the free energy of oxygen binding (log $P_{50}$) and the cross-link span in the product. This was observed where the proteins were cross-linked between β-Val-1 of one chain and the β-Lys-82 of the other chain (Jones et al. 1993). In contrast, where the cross-link is between two β-Lys-82 sites, a positive correlation between $P_{50}$ and bridge length is observed but the variation is small. Crystallographic analysis by Schumacher and Brennan showed that the cross-link prevents full relaxation upon oxygen binding, with longer links leading to more relaxation (Schumacher et al. 1995, 1997). These studies provide chemical knowledge required to design and adapt Hb to a desired P50.

### *10.4.6 Trifunctional Reagents: The "Spare Tire" Approach to Affinity and Conjugation*

The reagents that have been used to react regioselectively with amino groups of the protein are either dibromosalicylate esters or acyl phosphate monoesters. While both react rapidly with amino groups of the protein, they also react with water in the protein environment. This is the main drawback of the ester-based reagents. For a bi-functional cross-linker, a complete protein cross-link requires reaction to occur at two sites on the cross-linker. If one is hydrolyzed by water it is

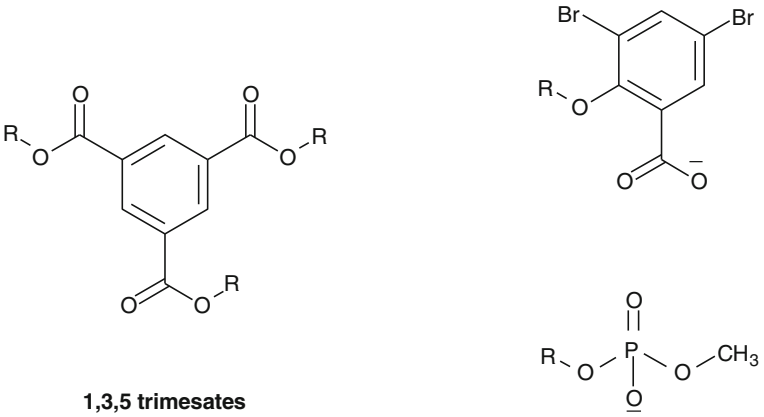


**Fig. 10.7** Tri-functional reagents can be activated either with DBS groups or with acyl phosphates. Both reagents can react with haemoglobin, cross-linking the tetramer efficiently, and even leaving a third electrophilic site available for other reaction

no longer reactive; only one site can react and this effectively prevents formation of a cross-link. This leaves a protein in a state that can be readily dissociated into the undesirable dimers.

As a partial solution to the problem, we reasoned that reagents with more than two sites could produce a cross-link even after one hydrolysis has occurred. Our most effective approach utilized derivatives of 1,3,5-trimesic acid. Both the tris-acyl phosphate monoester (Wodzinska et al. 1991) and tris-dibromosalicylate esters of trimesic acid (Kluger et al. 1992) were prepared and both reacted with Hb to produce cross-linked protein with high efficiency (Fig. 10.7). Both materials provide an efficient route to efficient production of cross-linked Hb. In additional, the remaining ester can provide a site for conjugation and coupling (Kluger et al. 1992; Kluger and Song 1994). In fact, the remaining ester can be utilized to couple two Hb tetramers together to produce bis-tetramers.

## 10.5 Inter-Molecular Cross-Linking (Oligomers, Polymers and Bis-Tetramers)

As we noted earlier, the vasoactivity attributed to the circulation of cross-linked Hb tetramers suggested that they are small enough to extravasate through channels in the endothelium where they scavenge NO. Avoiding extravasation would effectively remove the scavenger from the area where NO resides. Since NO is a signal that is amplified by enhancing the reactivity of guanyl cyclase, the muscles surrounding a blood vessel would be sensitive to even small changes in concentration of NO (Murray et al. 1995; Gukovskaya and Pandol 1994). Based on this

proposition, it is reasonable to propose that a safe and effective HBOC must be significantly larger than an Hb tetramer.

Kim-Shapiro, Schechter and Gladwin have considered the localization of NO along the endothelium in connection with the ability of acellular Hb to induce vasoconstriction (Kim-Shapiro et al. 2006). They proposed that the key features that prevent Hb within red cells from scavenging NO are associated with circulatory flow properties that control proximity to the endothelium. The relatively small protein travels in several directions within the overall circulatory flow while cells are subject to central laminar flow. How much larger than an Hb tetramer does an HBOC have to be in order to have flow properties that minimize scavenging of NO? While this cannot be predicted, evidence suggests that species that are only about twice the size of the tetramer can fulfill the criterion.

### *10.5.1 Glutaraldehyde Polymerized Hb: Chemistry*

In general protein modification, a reasonable place to start has been to use simple reagents that are known to react with protein side chains. Aldehydes been used as reaction sites for bi-functional and multi-functional reagents that combine with amino groups of the side chains of proteins (Habeeb 1967; Habeeb and Hiramoto 1968; Eike and Palmer 2004). Aldehydes react reversibly with amino groups, forming carbinolamines. These are unstable and lose water to produce the more stable imine (Schiff's base). In order for the product to be stable in circulation, the imine can be reduced to an amine by addition of an exogenous hydrogenation reagent. The best reducing reagent is chosen so that it does not otherwise affect the protein. Sodium borohydride as well as sodium cyanoborohydride and borane have been successfully implemented as reducing agents (Eike and Palmer 2004). While the reduction step adds complexity to the cross-linking process, it assures the permanent stability of the potential HBOC.

In considering aldehyde-based reagents for chemically stabilizing Hb tetramers, glutaraldehyde has typically been the reagent of choice. Glutaraldehyde had been widely used to keep multi-subunit proteins from dissociating, and once reduction with sodium cyanoborohydride is complete, provides functional products without creating toxic by-products (Habeeb 1967; Habeeb and Hiramoto 1968; Eike and Palmer 2004). Reactions of solutions of Hb with glutaraldehyde and subsequent reduction should produce materials that will not dissociate. In addition, during the cross-linking process, reactive anionic species additives were included in order to alter the $P_{50}$ of Hb to be similar to that of red cells in blood ($P_{50} = 26$ torr). However, one important consideration that makes the use of the reagent less appealing is that the chemical structure of glutaraldehyde is much more complex than the name implies. Glutaraldehyde undergoes oligomerization to an extent that makes a reacting solution heterogeneous (Migneault et al. 2004) (Fig. 10.8). Furthermore, the reactions of the species in the mixture are also not regioselective within the protein.

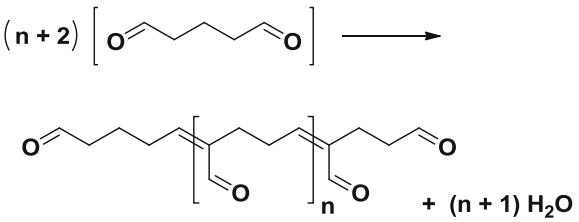


**Fig. 10.8** The chemical reagent, glutaraldehyde, polymerizes with itself to produce oligomeric versions of the reagent. This makes the reagent impure, introducing chemical cross-links within the protein that are of different lengths

As a result, solutions of stabilized Hb from reactions with glutaraldehyde are considered heterogeneous (a mixture of different products) and a separation technique such as size-exclusion chromatography is required to remove species whose size is either too low (smaller than tetramers) or too large (oligomers of multiple tetramers). In this type of Hb modification, a mid-sized assembly meets the overall requirements. Hb modified by glutaraldehyde is not only heterogenous in size, the reaction sites are also heterogenous with cross-links between various residues, including lysines, cysteines, histidines, and tyrosines (Habeeb and Hiramoto 1968). An obvious problem with working with such mixtures is that their clinical properties are likely to differ. If there is a positive outcome, the useful components are mixed among others that are probably nonfunctional. In the event of an unacceptable clinical outcome, with such a mixture it would not be possible to know which component caused the difficulty—making improvements impossible from such a mixed product.

### *10.5.2 Glutaraldehyde Polymerized Hb: Clinical Studies*

There were two commercial products previously in development that utilize glutaraldehyde polymerized bovine or human Hb. Both these products have been tested in clinical trials. These Hb polymers have a significant loss of allosteric cooperativity, and the modified Hb's p50 is typically right shifted and depends on the amount of glutaraldehyde used. The products derived from bovine Hb are Hemopure (HBOC-201), developed for human use, and Oxyglobin (HBOC-301) (BioPure, Cambridge, MA), developed for veterinary use. Both products are heterogenous mixtures of variously sized oligomers, ranging from 130 to 500 kDa (Jahr et al. 2008).

Hemopure (HBOC-201) has been extensively studied in multiple animal models and human clinical trials (Chen et al. 2009; Jahr et al. 2007; Pearce and Gawryl 2003). There are conflicting results that indicate that while vasoconstriction has been observed in a large number of studies in both animals and humans (Botzlar et al. 2002), likely resulting from depletion of NO concentrations after Hemopure infusion, other studies failed to observe vasoconstriction or a decrease

in NO concentration (Knudson et al. 2003). Nonetheless, the rapid formation of met-Hb (an indicator for Hb oxidation by interaction with free NO) was also observed in various animal and human settings (Jahr et al. 2008; Chen et al. 2009; Gould et al. 1992). In 2009, after a series of regulatory problems, Biopure filed for bankruptcy and its assets were purchased by OPK Biotech. Neither Hemopure nor Oxyglobin are currently available.

Northfield Laboratories Inc. (Evanston, IL) produced a glutaraldehyde-polymerized human Hb that is produced by reacting the Hb with pyridoxal 5′-phosphate before glutaraldehyde polymerization. This addition of pyridoxal 5′-phosphate, an allosteric analogue of 2,3-DPG, increases the p50 value from 18–22 to 28–30 mm Hg (Gould and Moss 1996; Gould et al. 1992).

### *10.5.3 O-Raffinose Polymerized Hb*

A novel aldehyde-based reagent was developed by Hemosol Inc. for producing their HBOC "HemoLink" that was the subject of extensive clinical trials. The cross-linking agent, "O-Raffinose", is a polyaldehyde that is produced by periodate oxidation of raffinose, a trisaccharide (Hsia et al. 1992; Ali et al. 1997; Eike and Palmer 2004). The reaction of raffinose with periodate is shown in Fig. 10.9. The oxidation produces numerous aldehyde functional sites on the oxidized product (Lieberthal et al. 1999). The multiple reaction sites of the reagent mean that each reaction combination (reagent aldehyde reacting with a protein amino group) would give a unique product, leading to a complex array of products that are stabilized in the Hemosol process by reduction with a borane derivative.

### *10.5.4 PEG Conjugation*

The conjugation of chains of polyethylene glycol to Hb is a straightforward process that leads to a large increase in the volume of the combined species (Nucci et al. 1996; Gombotz and Pettit 2000). The materials, generalized as "PEG-Hb",

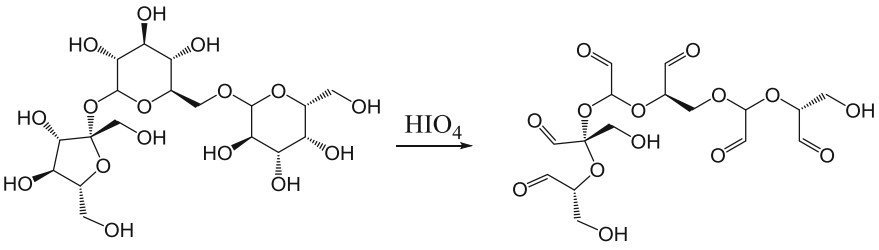


**Fig. 10.9** Raffinose is oxidized with periodate to produce a material with a large number of reactive aldehyde groups

have been reported to elicit minimal vasoactivity (Vandegriff et al. 2003). A product with multiple PEG chains conjugated to Hb is the basis of Sangart's MP-4, a development initially led by Winslow and Vandegriff (Vandegriff et al. 2003). Acharya has provided a detailed overview of the preparation and functional properties of PEG-Hb conjugates (Hu et al. 2005). The size and shape necessary to avoid extravasation and scavenging of NO that is accomplished by adding PEG comes with a serious cost—the added weight provides no additional capacity to carry oxygen.

### *10.5.5 Cross-Linked Bis-Tetramers: Chemistry*

The challenge to enlarge the protein while increasing oxygenation capacity in-proportion ideally could be achieved by connecting cross-linked tetramers to one another. The general approach is summarized in the Fig. 10.10.

Our first attempt to develop a reagent that would fit this strategy used a connector that is based on the chemical functionality of PEG, an oligomeric derivative of ethylene glycol. However, reaction of this material with Hb did not give a product that had more than one tetramer linked (Paal et al. 1996; Kluger et al. 1999). Instead, the connecting chain appeared to fold onto itself and all reactions were within the same tetramer. This could obviously be avoided if the connecting chain cannot fold.

Based on the hypothesis that an effective reagent must have a rigid connector between the reactive leaving groups, we prepared a material where the linker is derived from 5-amino-isophthalate as the protein reaction site and terephthalate as the connecting core (Kluger et al. 1999). The linker is then unable to fold and the reaction will produce a material that is a symmetrically connected assembly of two tetramers (Fig. 10.11). We refer to the resulting protein assembly in general as being a "bis-tetramer" to make clear its origins and general structure. Others have referred to such a structure an "octamer".

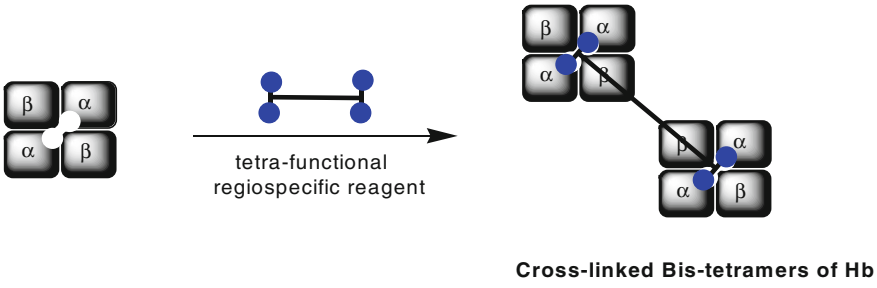


**Fig. 10.10** A tetrafunctional cross-linker that is designed with regiospecific directing groups (depicted in *blue*) will react with two separate haemoglobin tetramers at a specific cavity, while tethering the two proteins together. These Hb derivatives are cross-linked bis-tetramers of haemoglobin

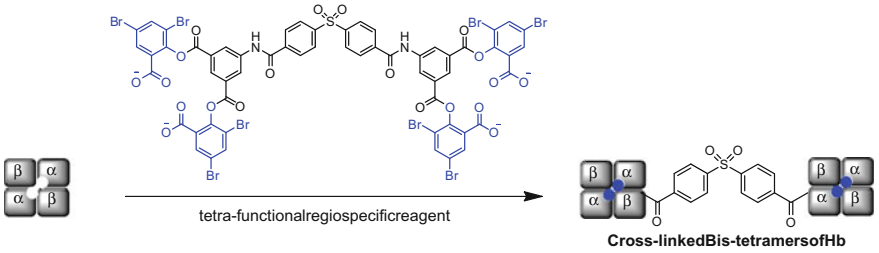


**Fig. 10.11** Reaction of haemoglobin with a tetrafunctional cross-linker containing four DBS directing groups produces cross-linked bis-tetramers of haemoglobin

The initial haemoglobin bis-tetramers produced from this reaction proved to be site-specific and pure, but the physical properties were found to be unlikely to be those of a successful product, due to the relatively low coperativity in oxygen binding ($n_{50} \sim 1.8$). Using chemical modelling to visualize the reagent in a 3D space, we realized that replacing one $sp^2$ atom with an $sp^3$ along the main bridge would reduce the length of the span between tetramers, forcing interactions between side chains of the tetramers that could be manifested as an increase in cooperativity (Hu and Kluger 2008). The example below meets the structural criteria with $n = 2.7$.

Further extension of this coupling strategy can produce higher order assemblies of tetramers, essentially making polymers of Hb. The cross-linked ester that results from reaction of Hb with trimesoyl tris(3,5-dibromo salicylate, TTDS) can react with multifunctional nucleophiles to give dendrimeric arrays, giving larger products (Kluger and Zhang 2003). Another approach is to add PEG chains to the bis-tetramer to further increase the size (Lui and Kluger 2009).

### *10.5.6 Cross-Linked Bis-Tetramers: Pre-clinical Studies*

The Zapol group has recently evaluated the potential of an Hb bis-tetramer (BT) and its PEGylated derivative (BT-PEG) as HBOCs (Lui et al. 2012). They studied the effects of administration of both materials on the blood pressure of normal and diabetic mice. The latter have endothelial dysfunction and are particularly sensitive to scavenging endogenous levels of nitric oxide. The diabetic mice thus serve as a sensitized model to evaluate the hemodynamic effects of NO-scavenging by HBOCs. In their report they compare the systemic vasoconstrictor effects of both bis-tetramers (BT) and PEGylated bis-tetramers (BT-PEG) in awake and anesthetized mice and find that systemic vasoconstriction is not produced by these compounds (as compared to injections of murine Hb). Also, infusion into diabetic db/db mice exhibiting endothelial dysfunction demonstrate that infusion of either BT or BT-PEG does not alter systemic blood pressure. Since a major drawback of traditional HBOCs are their vasoconstrictive effects (Ryter and Otterbein 2004;

Jahr et al. 2007; Buehler and Alayash 2004), this pre-clinical study demonstrates that BT-PEG may have many of the necessary qualities required for producing a safer and functional oxygen carrier.

### *10.5.7 CuAAC Coupling*

The preliminary success of Hb bis-tetramers in pre-clinical animal models has prompted us to develop chemical strategies that improve the production yield of Hb bis-tetramers. An alternative strategy to creating Hb bis-tetramers is to combine the cross-linking reaction with a highly efficient bio-orthogonal coupling reaction. A bio-orthogonal reaction utilized chemical groups that are not found in biological systems and must be synthetically prepared. The two bio-orthogonal groups seek each other out within a biological media, and react specifically with each other. In a first attempt we employed the use of the highly effective copper-catalyzed azide-alkyne coupling. First, haemoglobin must be activated with an azide, one of the bio-orthogonal groups. This can be achieved easily by incoporating the azide *into* the chemical cross-linking reagent (Azide-Hb, Fig. 10.12). Haemoglobin is then activated with the azide and cross-linked in a single step (Buehler and Alayash 2004). A bi-functional linker containing two alkyne groups (the complementary bio-orthogonal groups) is then chemically synthesized and added into the reaction system (bis-alkyne, Fig. 10.12). In the presence of Cu(I), the azide reacts in a specific and rapid cycloaddtion reaction with bis-alkyne to form triazole rings that is made up of the sum of the two reactants (Foot et al. 2009). In order to form

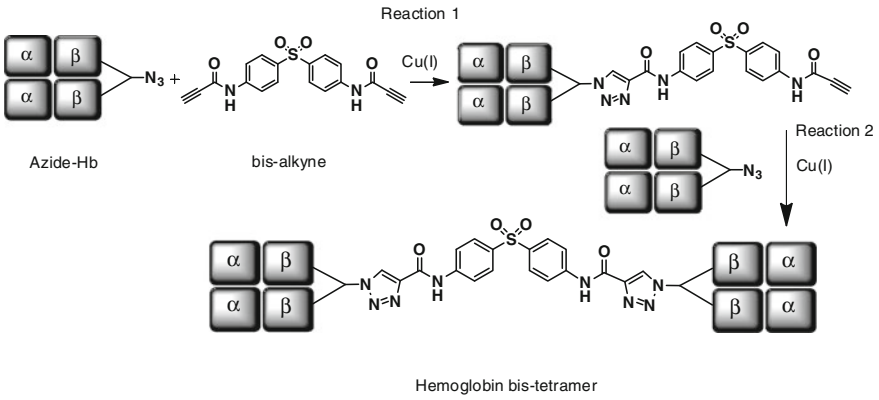


**Fig. 10.12** Using bio-orthogonal chemistry to introduce couple two haemoglobin tetramers together. Azide activated cross-linked haemoglobins (Azide-Hb) react with a bifunctional alkyne (bis-alkyne) through a copper catalyzed "click" reaction (Lutz and Zarafshani 2008). The initial coupling (Reaction 1) is slow due to the insolubility of the bis-alkyne. Once the first coupled product is formed, the second coupling step (Reaction 2) is much faster, producing haemoglobin bis-tetramers

a bis-tetramer, cross-linked tetramers containing the azide must react sequentially with the two alkyne groups of a bis alkyne. This can only work if the second reaction (Reaction 2, Fig. 10.12) is faster than the first (Reaction 1, Fig. 10.12). However, one might initially presume that there is no reason for any distinction between the two steps—all the initial azide could react with the bis-alkyne before a second reaction occurs, leaving the final product to be that from Reaction 1.

Instead, since the rate of a reaction depends on the concentrations of the reactants in the same phase, when the reaction is carried out in water, the bis-alkyne has very low solubility and reacts relatively slowly at the interface of the solid with water. In contrast, after the first reaction, the intermediate alkyne is attached to Hb and this alkyne is therefore present at a much higher concentration in the aqueous phase than is the bis-alkyne reagent because of its low solubility. We have now optimized this bio-orthogonal approach to producing Hb bis-tetramers (Foot et al. 2009; Kluger et al. 2010; Yang and Kluger 2010), and shown that it is possible to get Hb bis-tetramers with up to 50 % final yields. Given the benign hemodynamic effects of the first generation Hb bis-tetramers, we are optimistic that continued research in this area of Hb coupling will guide us towards developing new HBOCs that are safe to use.

## 10.6 Conclusions

Selective and efficient chemical reactions can be used to produce stabilized Hb tetramers as HBOCs in highly pure quantities. However the adverse clinical observations of many of the tested HBOCs indicated that these species will be not be suitable for administration into patients. New chemical procedures of enlarging the protein complexes while retaining specific modifications may be the first step towards developing new HBOCs that are safe and functional. In particular, the addition of PEG chains and the formation of assemblies of tetramers using more complex but efficient reactions has produced materials that have shown promise of being both safe and effective based on studies with animals. The possibility of the long-sought benefits projected for HBOCs should remain a valid prospect as the properties of these and other materials based on expanded knowledge of the complexities of the challenge are revealed and solved.

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# Chapter 11
# Design of Nonhypertensive Conjugated Hemoglobins as Novel Resuscitation Fluids

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## 11.1 Introduction

Grafting of molecular chemical entities, i.e. chemical modification of proteins and enzymes, has been the platform for biochemists for a customized taming or improvement of their properties. These new chemical entities could be small molecules or polymers, either naturally occurring or synthetic. The term conjugation generally refers to the grafting of polymers to proteins and enzymes to achieve such an objective. The advances in molecular cloning have introduced site directed mutagenesis for taming the molecular properties of proteins, which has evolved as the alternative approach to achieve the same objective. The de novo design of the protein/enzymes with customized structure/conformation and function has been the utopian dreamland. The integration of the site directed mutagenesis with the conjugation chemistries appears to be emerging as a powerful platform for design of novel peptide/protein therapeutics for very well defined clinical applications. These molecular approaches are classified together as Protein Engineering. However the use of the term "protein engineering" implies that the protein chemist who engages in these activities have fully understood the cross correlation of the structure and function of molecules that he/she is reengineering and can predict outcome with a high degree of certainty. This certainly is not the case.

The protein engineering has been, essentially an exercise of introducing knowledge based structural changes into the protein/enzymes and delineating the functional consequences. This has certainly been the case for designing and development of hemoglobin based oxygen carriers (HBOCs). The interactions involved in vivo in particular between the transport of oxygen by Hb, delivery of the

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oxygen to the tissues in desired levels, autoregulation of systemic circulation, microcirculation and NO homeostasis, etc., were not recognized until recently. Accordingly, the delineation of these aspects and integration of these concepts into the design of HBOC has been frustrating. The conjugation of polyethylene glycol (PEG) to bovine Hb advanced by Enzon has emerged as a primary game changing molecular event to the field of transfusion, in particular design of new resuscitation fluids (Cabrales et al. 2007, 2008; Tsai and Intaglietta 2002). PEGylated Hbs, in particular the hexaPEGylated Hb (EAF P5K6 Hb), and its many structural analogues designed at Albert Einstein College of Medicine (Einstein) and investigated at the University of California, San Diego (UCSD) have played a pivotal role in understanding the PEGylation induced changes in the solution, molecular and functional properties of Hb. The correlation between the structure of PEG shell and the plasma expander like properties of PEGylated proteins is now being appreciated. PEG Hbs have turned out to be a new class of Hb derivatives, oxygen carrying plasma expanders. Hemospan® (MP4), a prototype of Einstein EAF P5K6 Hb has been extensively pursued by Sangart as an oxygen therapeutic and has shown that EAF PEG Hbs, at the concentrations attempted so far, have little or no in vivo toxicity.

This chapter is focused on discussions of the development of various conjugated Hbs as a new class of potential HBOCs with particular emphasis on the correlation between conjugation and the microcirculation. The primary focus of this Chapter is to discuss development of this class of molecules and how all the studies lead to the recognition of the significance of engineering plasma expander like properties to attenuate the in vivo hypertensive activity of acellular Hb. However, PEGylated Hbs have been discussed in greater details in particular emphasizing their current limitations and the future directions to develop PEG Hb as supra perfusionary resuscitation fluid(s) with functional properties optimized for both transporting and delivering oxygen. Some aspects of site directed mutation of Hb carried out in collaboration with Chien Ho at Carnegie Mellon University to introduce Cys at some unique positions on the surface of molecule as targeted sites for PEGylation with or without extension arm facilitated PEGylation is also discussed. We advanced the possibility that unique sites exist on the surface of Hb for PEGylation that impacts the least on the escape of oxygen from the Hb molecules through PEG shell and thus facilitates a better tissue oxygenation. Mapping these sites and targeting the PEGylation to these sites and developing a molecular level understanding of this phenomenon remains the future challenge in advancing PEG Hbs as novel oxygen therapeutics.

## 11.2 Conjugation of Synthetic or Natural Polymers to Proteins

The term conjugated protein is used in protein chemistry to describe proteins that contains constituents other than naturally occurring amino acids, for example Hb. Hb is a tetrameric protein, consisting of four copies of polypeptide chains, two

copies each of $\alpha$ and $\beta$ globins, and each copy of the globin chain carries one prosthetic group, heme. In practice, however, the chemical process of attaching non-protein materials to proteins has generally come to be referred to as conjugation.

The earliest known attempt to modify proteins using polymers was at NIH in the sixties by Christian B. Anfinsen (Epstein and Anfinsen 1962). Anfinsen's group developed new approaches to conjugate homopolymeric strands of unnatural amino acid strands on the surface amino groups of bovine pancreatic ribonuclease to delineate the influence of these conjugated homopolymers on the reversible unfolding and refolding of reduced ribonuclease. A systematic study of conjugation of polymers to proteins began in the seventies when Abuchowski and Davis conjugated synthetic polymer, polyoxyethylene of defined molecular sizes to proteins, now referred to as PEGylation (Abuchowski and Davis 1979; Abuchowski et al. 1977; Savoca et al. 1979) and the whole field is referred to sometimes as "PEGnology". Since then modification of proteins with other synthetic polymers and other biopolymers has also been advanced. These included the use of polystyrene, polylactic acids, dextran, starch, polyoxy propylene, copolymers of oxypropylene and lactic acids, etc. for generating conjugated proteins. In the most simplistic terms of conjugation chemistry, even protein oligomerization (polymerization) can be considered as a conjugation protocol, as proteins by themselves are polymers of amino acids.

## 11.3 Strategies to Attenuate the in vivo NO Scavenging by Hb to Overcome Hypertension

The primary limitations of using acellular Hb as an oxygen carrier have been the nephrotoxicity and NO scavenging mediated vasoconstriction of arteries and arterioles (Alayash 2010; Elmer et al. 2012; Jahr et al. 2012; Chen et al. 2009; Buehler and Alayash 2008; Winslow 2006, 2008; Sampei et al. 2005). Overcoming the nephrotoxicity was simple. Introducing intramolecular crosslinking between the $\alpha\beta$ dimers of Hb completely neutralizes the dissociation of tetrameric Hb into $\alpha\beta$ dimers and accordingly prevents Hb filtering through kidney. This approach completely overcomes the nephrotoxicity. Intramolecularly crosslinked Hbs (without impacting the molecular dimension) are essentially free of nephrotoxicity.

However, such molecules still exhibit significant level of in vivo hypertensive activity. This activity of intramolecularly crosslinked Hbs has been attributed to the intrinsic NO scavenging by Hb. A number of other intramolecularly crosslinked Hbs, where in the interdimeric crosslink introduced is inside the central cavity, and with lower oxygen affinity than the control Hb have also been designed as potential blood substitutes (Walder et al. 1994; Benesch and Benesch 1981; Bucci et al. 1996). However, all intramolecularly crosslinked Hbs exhibit vasoconstrictive activity in experimental studies and are all hypertensive when the animal is infused with these products. More, recently it has been argued by

Winslow and his colleagues (Winslow 2003; Tsai et al. 2003) that the earlier paradigm of generating low oxygen affinity Hbs (with an oxygen affinity comparable to that of Hb in erythrocytes, with DPG present) by chemical/genetic approaches as a not well conceived concept for the design of blood substitutes as such molecules will unload their oxygen in arteries and arterioles to induce autoregulation dictated vasoconstriction. Winslow has concluded αα-fumaryl Hb was a molecule destined to fail as blood substitute (Winslow 2000). A new class of Hbs referred to as outside the central cavity intramolecularly crosslinked Hb have been designed, wherein a PEG based bifunctional crosslinker (the spacer arm of the crosslinker is flexible) is used to generate this class of molecules (Acharya et al. 1996). A high oxygen affinity Hb, Cys-93($\beta\beta'$)-succinimido phenyl PEG 2000 Hb was developed by Acharya et al. (Manjula et al. 2000). The vasoconstrictive activity of the high oxygen affinity of αα fumaryl Hb and Cys-93($\beta\beta'$)-succinimido phenyl PEG 2000 Hb have been compared in extreme hemodilution models and exhibited comparable vasoconstrictive activity as reflected by the concomitant reduction of functional capillary density.

The approaches designed and developed to attenuate the NO scavenging activity of Hb and generate Hb based oxygen carriers can be classified into three broad categories: (i) enhancing the molecular size, (ii) reducing the intrinsic NO scavenging activity of Hb by site directed mutagenesis (Eich et al. 1996; Doherty et al. 1998), and, (iii) supplementing the blood substitutes with either NO generating particles (Cabrales et al. 2013) or loading the designed blood substitutes with covalently linked nitrosothiols that can release their NO in vivo thereby compensating to some degree the Hb NO scavenging activity. This chapter is focused only on strategies initially designed to increase the molecular dimension of Hb with an expectation that reducing the extravasation of Hb will reduce NO scavenging mediated hypertension. New classes of conjugated Hb molecules that exhibit a very large increase in hydrodynamic volume have been now identified as nonhypertensive derivatives of Hb, i.e. PEG Hbs. The conjugation of PEG to Hb increases the hydrodynamic volume of Hb with an efficiency that is nearly eight times higher as compared to the conjugation of a comparable mass of protein, and in doing so PEGylation of Hb engineers plasma expander like properties to the Hb molecule. The molecular aspects of taming the vasoconstrictive activity of Hb molecule by PEGylation are discussed here in detail.

### *11.3.1 Enhancing the Molecular Dimensions of Hb by Oligomerization*

Significant efforts have been focused on the low oxygen affinity human Hb, and αα-fumaryl Hb of Baxter (Walder et al. 1994) still remains the most potent Hb derivative in terms of the vasoconstrictive activity. Designing low oxygen affinity blood substitutes with enhanced molecular size (dimensions) has been initially

attempted by one-step oligomerization platform that introduces intra and intermolecular crosslinking using glutaraldehyde. Chang introduced the oligomerization concept (Chang 1964, 1971), and since then many new bifunctional crosslinkers have been advanced for the oligomerization of Hb (Bunn et al. 1969; Payne 1973; Marini et al. 1989; D'Agnillo and Chang 1998; Hai et al. 1999; MacDonald and Pepper 1994; Hai et al. 1994; Bakker et al. 1992; Dellacherie and Vigneron 1991; Zhang and Palmer 2010; Faggiano et al. 2010; Tarasov et al. 2007; Buehler et al. 2006; Scurtu et al. 2013; Berbers et al. 1991; Adachi et al. 1991).

#### 11.3.1.1 Glutaraldehyde Crosslinking Mediated Size Enhancement of Hb

The low oxygen affinity bovine Hb or low oxygen affinity chemically modified human Hb (pyridoxylated Hb) has been oligomerized by using glutaraldehyde. Hemoglobin Glutamer-250 (Hemopure®) of Biopure Corp/OPK Biotech and Polyheme® of Northfield were generated by glutaraldehyde mediated oligomerization approach. This approach distinguishes itself from the Baxter intramolecular crosslinking approach in that it attempts to attenuate both nephrotoxicity and vasoactivity of Hb by one chemical process, oligomerization. A one step intra and inter molecular crosslinking approach that achieves both oligomerization and reduces the oxygen affinity of human Hb was developed by Hemosol using oxidized raffinose (Dellacherie and Vigneron 1991; Scatena and Giardina 2001). This product is referred to as Hemoglobin Raffimer (Hemolink®). It should be noted all these oligomerized products have an average molecular size around 250K. The design strategy for this product is apparently flawed as reflected in the clinical trials and failure to obtain FDA approval. Nonetheless it should be noted that Hemoglobin Glutamer has been approved for veterinary use in US, and has been approved for human use in South Africa since 2001 and in Russia since 2010. It may be noted that Hemoglobin glutamer-200 (Oxyglobin®), a Biopure (now OPK Biotech) product has been is use for veterinary applications for some time in US.

Genetically intramolecularly crosslinked recombinant Hbs and their mutant forms have been generated to reduce the intrinsic NO scavenging activity Hb. One such form has been generated by oligomerization using glutaraldehyde. These studies revealed that oligomerized products have reduced hypertension (Doyle et al. 1999). This is consistent with the concept that attenuation of extravasation in vivo can induce beneficial effects in terms of Hb induced hypertension.

#### 11.3.1.2 Size Enhancement by Intermolecular Disulfide Crosslinking

Fronticelli and her colleagues (Bobofchak et al. 2003) advanced the novel genetic approach for generation intermolecular disulfide bridges into mutant forms of Hb. A recombinant hybrid hemoglobin molecule was designed using the human $\alpha$-subunit and the bovine $\beta$-subunit, with placement of surface cysteines to permit

disulfide bond polymerization between the tetramers. These mutagenetically introduced Cys are the targeted sites for the chemical oligomerization reactions, the thiol group of which facilitates the formation of inter tetrameric disulfide bonds. Three different molecular size forms of Hb Polytaur have been generated and all appear to be capable of reducing ischemia in the brain. This again suggests the beneficial influence of enhancing the molecular dimensions of Hb. The ability of these mutant Hbs to attenuate ischemia was shown to be independent of oxygen affinity of the product. The polymers formed here via intermolecular disulfide bonds are asymmetric molecules. Mutagenic approaches have also been developed to generate octameric forms of Hb that carries symmetrical inter tetrameric disulfide bonds (Vasseur-Godbillon et al. 2006).

#### 11.3.1.3 Zero Length Intermolecular Crosslinking

Site specific intramolecular crosslinking of deoxy Hb with in the $\beta\beta$-cleft using active esters of aliphatic dicarboxylic acids have also been advanced to generate low oxygen affinity Hbs for application as blood substitutes. $\beta\beta$-sebacyl human Hb generated by the reaction of the active esters of sebacic acid with the intramolecular crosslinks between the $\varepsilon$-$NH_2$ groups of Lys-82($\beta$) is a member of this class. DNX corp. in New Jersey has tried unsuccessfully to translate this reaction to recombinant human Hb expressed in transgenic swine and commercialize this product (Bucci et al. 1996). Pyridoxal phosphate based bifunctional reagents have been advanced by Benesches to introduce intramolecular crosslinks within $\beta\beta$–cleft of Hb lowering oxygen affinity. All these reactions have been carried out in the deoxy state. Manjula et al. (1995) introduced the use of sulfosuccinimidyl suberate and sulfosuccinimidyl sebasate as affinity directed intramolecular crosslinkers for generating an intramolecular crosslink within the $\beta\beta$ cleft of Hb in the oxy state. The negatively charges of sulfo groups in these crosslinkers facilitates the affinity directed noncovalent interactions of the crosslinkers at the $\beta\beta$-cleft in the oxy conformation of Hb prior to the formation of the crosslink. The $\beta\beta$-crosslinked low oxygen affinity Hbs are all vasoactive.

Bucci and his colleagues (Fronticelli et al. 1990; Mito et al. 2009) have achieved the polymerization of low oxygen affinity $\beta\beta$-sebacyl bovine Hb by a new intermolecular protocol referred to as zero length intermolecular crosslinking. Thus, this polymerization protocol is a two-step platform; the first introduces site-specific intramolecular crosslink into bovine Hb within the $\beta\beta$-end of the central cavity and in a second step non-specific intermolecular crosslinks are introduced between the surface $\varepsilon$-amino group of Lys residues of one Hb molecule and $\beta$ or $\gamma$ carboxyl groups of surface Asp or Glu residues (respectively) of another Hb molecule to form an isopeptide linkage between Hb tetramers. This approach is referred to as "zero length crosslinking" as there are no spacer arms involved in the intermolecular covalent bonds and involves the carbodimide mediated activation of surface carboxylates of Hb. This approach has led to the isolation of a product with a molecular radius of 25 nm (Matheson et al. 2002), with high

viscosity and a high oxygen affinity. It may be noted that high viscogenic materials have emerged as good vasodilators as well as plasma expanders (Cabrales et al. 2004; Tsai et al. 1998; Acharya et al. 2011; Sriram et al. 2012). Consistent with the high viscosity of the material, the zero length crosslinking results in attenuation of the in vivo hypertensive activity of Hb, and the product is essentially nonhypertensive. A cross correlation of the molecular dimensions with the oligomerization induced increase in viscosity is not available at this stage, accordingly it is not clear whether this attenuation of in vivo hypertensive activity of Hb is a consequence of enhanced molecular dimension or induced viscosity, or a combination of both. It has also been argued that the very large molecular dimensions of the Zero crosslinked Hb do not extravasate and hence do no scavenge NO, and are hence vasoinactive and supplementation of NO through NO particles has been advanced as new approach to overcome the vasoactivity of blood substitutes (Cabrales et al. 2013). On the other hand, the studies of Intaglietta and his colleagues (Cabrales et al. 2004), have well established that high viscosity materials like dextran 500 and alginate are supra perfusion materials, however the threshold viscosity of the resuscitation fluid for inducing vasodilatation has not yet been clearly delineated.

The polymeric form of sebacyl bovine Hb generated by zero length crosslinking approach is to be developed by Oxyvita as oxygen carrying resuscitation fluid for veterinary use. A protocol for storing this material as lyophilized product has been advanced by (Harrington et al. 2011). It is not clear whether this is the reemergence of the concept of avoiding the extravasation by enhancing the molecular dimension to design vasoinactive blood substitute (Cabrales et al. 2013) or a chemical approach of enhancing the molecular dimension of Hb to the region of 25 nm for engineering Viscogenicity to the samples of Hb for facilitating NO production to design blood substitute with the benefit of attenuation of in vivo hypertensive activity of the molecule (Cabrales et al. 2004; Tsai et al. 1998; Acharya et al. 2011; Sriram et al. 2012).

A common feature of all three oligomerization protocols discussed above used for enhancing the molecular dimension of Hb is that multiple crosslinks had to be introduced between Hb molecules. This situation arises in view of the absence of intramolecular crosslinking (inter dimeric crosslinks) in the starting tetramers used for oligomerization with the exception of the $\beta\beta$-sebacys crosslinked Hb. Even in this case multiple crosslinks have been engineered to generate the material with a molecular radius of 25 nm. All the oligomerized molecules are expected to be essentially in a globular shape with very limited flexibility, the packing density of the polymer will not be changed and same as the protein molecule. On the other hand all the high viscogenic materials identified by Intaglietta and colleagues are not materials of high packing density (Cabrales et al. 2004; Tsai et al. 1998; Acharya et al. 2011; Sriram et al. 2012), and are expected to minimize the damage impacted to the endothelium in inducing shear stress (see Sect.11.3.3 for additional discussions).

#### 11.3.1.4 Outside the Central Cavity Intramolecular Crosslinking

All intra and intermolecular crosslinked Hbs discussed above introduce a very high degree of rigidity to the polymer molecule generated. An outside the central cavity intramolecular crosslinking of Hbs (Acharya et al. 1996), a novel intramolecular crosslinking approach that conserves the flexibility of the molecule for the intra and inter-dimeric interactions of the Hb within its quaternary structure, has been developed at Einstein. In this new approach, PEG based bifunctional reagent is used to crosslink the $\alpha\beta$-dimers of Hb wherein the long PEG chains serve as the spacer arm between two functional ends of the reagent. The initial choice of the reagent was PEG based bis maleimides. The PEG chains of the reagent after introducing an intramolecular crosslinking into Hb remains outside the Hb molecule, in contrast to the situation seen with the conventional intramolecular crosslinkers wherein the spacer arm remains within the central cavity of Hb tetramer. In this new approach, the crosslink is expected to conserve the complete intrinsic flexibility of the dimeric interactions of Hb as opposed to decreasing the flexibility through central cavity intramolecular crosslink. In one such molecule, the crosslinking is targeted to Cys-93($\beta$) using bis maleimidophenyl PEG 2K. Site specific Extension Arm Chemistry, (see below) has been now adapted to this new approach to introduce an outside the central cavity crosslink into Hb on the surface Lys residues, and have the Cys-93($\beta$) reversibly protected through the formation mixed disulfide with thiopyridine.

The quantitative efficiency of Bis Mal PEG 2K to introduce outside the central cavity crosslinks into Hb is a very surprising aspect of this intramolecular crosslinking reaction. Interactions of the PEG chain of the monofunctionally modified Hb by the bifunctional PEG reagent on to the molecular surface can dictate the high efficacy of the intramolecular crosslinking, a direct mechanism. The high efficacy of crosslinking could also be explained as a consequence of the generation of intermolecularly crosslinked species of Hb tetramer first and the subsequent rearrangement of the asymmetric intermolecularly crosslinked octamers of uncrosslinked Hb tetramers into symmetrical intramolecularly crosslinked Hb and uncrosslinked Hb (Fig. 11.1), as an indirect mechanism. If the indirect mechanism is the predominant pathway, Bis Mal PEG 2K crosslinking reaction with an intramolecularly crosslinked Hb, for example $\alpha\alpha$-fumaryl Hb, will be a novel way to generate asymmetric octomeric and higher linear ologomeric forms of Hb. However, this indirect approach of generating intramolecularly crosslinked Hb appears to be only a minor pathway/mechanism for generating the outside the central cavity crosslinking. The efficacy of engineering this intramolecular crosslinking into $\alpha\alpha$-fumaryl Hb by the Bis Mal PEG is a function of the length of the PEG chain of the bifunctional reagent, and increases as the chain length decreases. With a short PEG chain like PEG 600, the formation of an octomer (a dimer of $\alpha\alpha$-fumaryl Hb) and of higher oligomeric forms is the maximum. If the PEG chain is further shortened to PEG 175, the formation of inter molecular crosslinks and formation of higher molecular weight forms of Hb is essentially inhibited. The maximum length that can be spanned by this short PEG chain of the

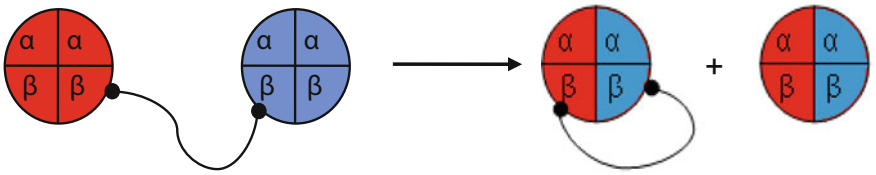


**Fig. 11.1** The schematic representation of the indirect molecular mechanism for the formation Bis Mal PEG 2K based outside the central cavity intramolecular crosslinking of uncrosslinked Hb. Bis Mal PEG 2K with long spacer arms can modify the two Hb termers monofunctionally resulting in the formation of an octomer. Thus each Hb tetramer in the octomer is asymmetrically modified, is thermodynamically unstable and readily segregates to general symmetrical outside the central cavity intra molecularly crosslinked Hb and uncrosslinked Hb. When the spacer arm of the Bis Mal PEG 2K is shorter than the distance between the thiol groups of Cys-93($\beta$) in the Hb tetramer, such segregation is not possible. Accordingly by using the intramolecularly crosslinked Hb, and using bis maleimides with very short PEG spacer arm, the intermolecular crosslinking of intramolecularly crosslinked Hb will be favored. In the absence of intramolecular crosslinking, multiple intermolecular crosslinks needs to be introduced to stabilize the oligomer

crosslinker is shorter than the distance between the thiols of two Cys-93($\beta$) in the Hb tetramer molecule.

#### 11.3.1.5 Extension Arm Chemistry Facilitated Intra and Intermolecular Crosslinking

The unique molecular principles that dictate the formation of the outside the central cavity intramolecular crosslinks between the thiols of the two Cys-93($\beta$) residues of tetrameric Hb by Bis Mal PEG has formed the basis of the design and development of a new Extension Arm Chemistry Facilitated, Bis Mal PEG based intermolecular crosslinking approach for oligomerization of intramolecularly crosslinked Hb (Fig. 11.1). In this new approach for Hb (or other proteins for that matter) oligomerization protocol, the principles of extension arm chemistry (detailed discussion in Sect. 11.3.3), the chemical protocol to engineer new (extrinsic) functional groups on the molecular surface of proteins, has been integrated with the use of short flexible bifunctional bis maleimide PEG reagents (Li et al. 2006; Manjula et al. 2005; Acharya et al. 2005). In this two step oligomerization approach, flexible PEG spacer arm based intermolecular crosslinks are engineered between the newly introduced thiols of a thiolated intramolecularly crosslinked Hbs using Bis Mal PEG. These crosslinks are in contrast to the rigid and short crosslinks introduced using glutaraldehyde or zero length crosslinking approach practiced in the field. By limiting the number of crosslinks that can be introduced between the Hb tetramers the molecular shape of the polymer form can be dictated. When the number of intermolecular crosslinks between the two tetramers is restricted to one, a long ellipsoid shaped polymer is generated; when multiple crosslinks are introduced between two tetramers globular molecular shape

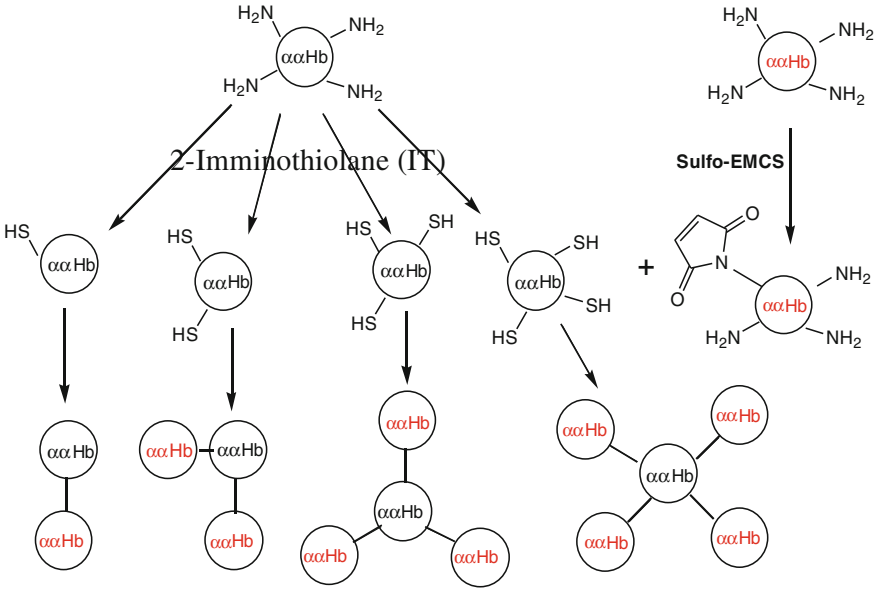


**Fig. 11.2** Schematic representation of a two-step complimentary extension arm chemistry based intermolecular crosslinking of intramolecularly crosslinked Hb. By controlling the extent of thiolation of αα fumaryl Hb in the first step, the molecular size enhancement of intramolecularly crosslinked Hb can be manipulated. The thiolated αα fumaryl Hb in the oligomer is represented by *black* and the maleimide (sulfo EMCS) modified Hb in the oligomer is represented by *red* color

is induced to the polymer generated. Extension arms with complimentary functional groups at their distal end, for example one set of tetramers carrying multiple copies of extension arms with thiols and another set of tetramers with a single copy of extension arm with maleimide (and in a significantly higher molar concentration) is a preferred strategy to generate a globular polymeric Hb and is depicted schematically in Fig. 11.2. It is anticipated that the ellipsoidal oligomers are with a higher flexibility and are expected to be more viscogenic materials than the globular forms, and hence are expected to better attenuate the in vivo hypertensive activity of Hb.

This approach of oligomerization of Hb tetramers with minimal number of intermolecular crosslinks between tetramers can only be practiced with intramolecularly crosslinked Hbs. The outside the central cavity crosslinking of Hb with Bis Mal PEG maleimide discussed above is targeted to Cys-93($\beta$). The modification of this thiol with maleimide increases the oxygen affinity and also induces nitrite reductase activity (Acharya et al. 2011). In an attempt to minimize the influence of outside the central cavity crosslinks on the oxygen affinity, this approach has been now modified to target such crosslinks to the surface ε-amino groups using extension arm chemistry. The thiol of Cys-93($\beta$) is protected as mixed disulfide of thiopyridine, and then this modified Hb is subjected to

thiolation at the ε-amino groups for targeted outside the central cavity crosslinking with Bis Mal PEG maleimide PEG 2K.

Oligomers with three to four copies of Hb have in general appears to have small but still noticeable impact in attenuating the vasoactivity of the parent molecule as reflected by the reduced mean arterial pressure as compared to αα-fumaryl Hb. The zero length crosslinking of intramolecularly crosslinked Hb has a very clear effect in attenuating the hypertensive activity of Hb. Interestingly, the molecular radius of the nonhypertensive zero length crosslinked ββ-sebacyl bovine Hb is around 25 nm. This is the range of the molecular radius of NO producing high viscosity plasma expander dextrans, for example dextran 500. However the packing density of dextran is significantly lower than that of the zero length crosslinked Hb, i.e., the flexibility of the two materials is very different. The increase in viscogenicity of the oligomeric forms of intramolecularly crosslinked Hb can also be modulated efficiently as a function of number of copies of tetramer in the polymeric molecule by modulating the pattern of intermolecular crosslinking of Hb to generate an ellipsoidal forms vs a globular forms (Fig. 11.2). The generation of an ellipsoidal form has not been advanced so far. Besides modulating the viscosity, the oligomerization approach to generate ellipsoidal forms can also readily facilitate the engineering of a lower packing density and higher structural flexibility to the polymer by varying the spacer arm PEG chain size and the tetramer number in the polymer. The relative advantages of this class of molecules over the Hb surface decorated with PEG chains (see Sect. 11.3.3 on PEG Hbs) needs to be pursued in the future to identify the design of the best oxygen carrying resuscitation fluids.

#### 11.3.1.6 Other Conjugation Approaches to Attenuate the Hypertensive Activity of Acellular Hb

Polymerizing Hb along with Superoxide dismutase and catalase has been advanced as a new strategy to overcome the toxicity of the acellular Hb by Chang and his colleagues (D'Agnillo and Chang 1998; D'Agnillo and Chang 1993). Synzyme has introduced polynitroxylation of αα-fumaryl Hb (Buehler et al. 2000), the most vasoactive molecule, as an approach to attenuate the in vivo vasoconstrictive activity of the molecule. The tempol groups conjugated are free radical scavengers and are thus superoxide mimetics, and this approach is in line with approach advanced by Chang. Though the polynitroxylation approach has been developed with αα-fumaryl Hb, the approach could be used with other oligomeric Hbs generated by any one of the approaches discussed above.

It may be noted that an Extension Arm Chemistry based approach for polynitroxylation has been developed at Albert Einstein College of Medicine, which is simpler, more quantitative than the Synzyme approach and the tempol conjugated to protein using this approach exhibits a SOD mimetic specific activity two to three fold higher than that of tempol conjugated without the use of Extension Arms (see Sect. 11.3.3 for additional details).

### *11.3.2 Conjugation of Multiple copies of Hb to a Natural or Synthetic Polymer*

Conjugation of multiple copies of Hb to a molecule of functionalized naturally occurring biopolymers, polysaccharides, as well as to synthetic polymer such as dendrimer is another approach for generating conjugated Hbs. In this approach a polyfunctional polymer carries multiple copies of covalently attached Hb molecules. However, there are no intra or intermolecular cross-links in Hb. In this approach the molecular dimensions of conjugated Hb generated will be significantly higher than the combined molecular dimensions of the Hbs and will include the dimensions of the polymer (the polyfunctional crosslinker). Since dextran is a significantly disorganized molecule with large molecular radius as compared to the protein of a similar molecular mass, the conjugates generated exhibits a significantly larger molecular dimensions and flexibility, and accordingly effectiveness of this approach in enhancing the molecular dimensions of Hb is far superior to the oligomerization approach.

Deoxytranslation approach has been pursued for a long time to generate new macromolecular forms of Hb (Styslinger et al. 2012; Eike and Palmer 2004). Starch and dextran of different molecular sizes have been derivatized in a way that introduces multiple copies of functional groups; and these are targeted for conjugation to Hb molecule. Since both starch and dextran have been in clinical use as plasma expanders, in principle this can be considered as the beginning of the overall concept of engineering plasma expanders like properties to Hb for generating nonhypertensive forms of Hb as oxygen therapeutics (Klett et al. 1992). However, the unique benefits of using dextran as the molecular vehicle to carry multiple copies of Hb molecules in terms of the microcirculatory advantages was not realized or appreciated earlier. Intaglietta and his team have exposed the fact that molecular and solution properties of decaPEGylated Hb of Enzon is very distinct as compared to other Hb derivatives designed as blood substitutes (Salazar Vázquez et al. 2008). Its properties has some resemblance to molecular and solution properties of dextran 70 and of hetastarch, the colloidal plasma expanders.

The chemical concept of conjugation of multiple copes of Hb to a naturally occurring polymer, dextran, was first advanced by Wong and his colleagues (Chang and Wong 1977). Dextran is a branched polysaccharide composed of glucose units. Thus in designing dextran conjugated Hb, if one uses the right molecular size of derivatized dextran as the polyfunctional reagent, for example dextran 70, the resultant dextranylated Hb is oxygen carrying resuscitation fluid.

#### 11.3.2.1 Conjugation of Hb to Poly Aldehyde Dextran

The periodate oxidation of vicinal diol and of amino alcohol to convert them into dialdehydes is a well-established protocol in protein chemistry. This approach has been translated for generating polyaldehydic dextran. Wang and his colleagues

(Chang and Wong 1977) used dextran polyaldehyde as the macromolecular reagent to conjugate multiple copies of Hb molecules to achieve oligomerization. In these the aldehyde functions are generated on the internal glucose units of dextran. Amino groups of Hb can react with these aldehyde functions of dextrans to generate dextran Hb conjugates, the aldimine (Schiff base) linkage generated between dextran and Hb is not stable, and has to be reduced to alkylamine linkages to gives the adducts. Unmodified Hb was used in the early studies and the product exhibited a high oxygen affinity. It is interesting to note that the viscosity of a solution containing 5 % Hb conjugate with dextran (Dx 20 kDa) is about 2.5–3.0 cp (Chang and Wong 1977) and this is slightly higher than viscosity of a 4 gm (based on Hb content) solution of EAF P5K6 Hb. Another dextran conjugated Hb has been generated by Wong (1988) that has a moderate oxygen affinity with p50 around 23 mm Hg, using intramolecularly crosslinked Hb generated by inositol-tetraphosphate dialdehyde modification instead of uncrosslinked Hb. Thus it is possible to manipulate the composition of dextran Hb conjugate to customize the oxygen affinity and the viscosity of the final product as desired, much in the same way as we have done with PEGylated Hbs (see Sect. 11.3.3, and Sect. 11.3.2.3).

#### 11.3.2.2 Functionalization of Dextran without Using Periodate Oxidation

Several alternate approaches have been developed to functionalize dextran at their hydroxyl groups with multiple copies of functional groups per molecule, for example, amino, carboxyl and aldehyde moieties (Bonneaux et al. 1981; Dellacherie et al. 1983; Baldwin and Chien 1988). Conjugation of uncrosslinked Hb to negatively charged dextrans, like dextran sulphates, dextran phosphates and dextran carboxylates has been found to generate conjugates with an oxygen affinity lower than that originally prepared by Wong. The approach of Dellicheri and Bonneaux (Bonneaux and Dellacherie 1995) to generate dextran with aldehyde groups has formed the basis for the generation of a new form of low oxygen affinity dextranylated Hb. The French group has generated a polycarboxylic dextran (Bonneaux and Dellacherie 1995). This functionalized dextran has been conjugated to Hb to generate isopeptide bonds between the carboxyl groups of polycarboxylic dextran and the amino groups of Hb using carbodimide. The amino terminal Val residues have been suggested to form covalent linkage between Hb and the functionalized polymer.

Hb conjugated to benzene tetracarboxylated dextran is referred to as Dex-BTC-Hb. This product exhibits a low oxygen affinity, and is probably the best-studied product of this class of conjugated Hbs (Quellec et al. 1994; Menu et al. 1994). Its oxygen affinity is close to that of RBC but its cooperativity and Bohr effect is lowered compared to unconjugated Hb. It is interesting to note that the NO binding activity of Hb has also been reduced in this molecule. The molecular aspect for this aspect of the conjugate is not readily apparent. Nonetheless, the molecule appears to have some vasoactivity as per some reports (Quellec et al. 1994;

Menu et al. 1994). Alayash and his colleagues (Jia et al. 2004) have noted that this class of dextran Hb conjugate maintains distinct rheological properties as compared to Hb and other plasma expanders and accordingly better microvascular function as well. Accordingly dextran Hb conjugate also induces a shear thinning effect on RBC, a phenomenon recently observed in the presence of EAF PEG albumin (see Sect. 11.3.3).

Bromodextran is another functionalized dextran that has been used to form dextran Hb conjugate by Dextro-Sang Corporation and this company is attempting to commercialize this product as blood substitute.

#### 11.3.2.3 Extension Arm Facilitated Conjugation of Hb to Functionalized Dextran

At Einstein, the Extension Chemistry based platform designed for introducing functional groups in desired numbers on Hb for targeted conjugation to functionalized PEG has been now translated to conjugate dextran to Hb as well. New platforms have been designed to generate EAF Dextran Hb conjugates, EAF dextran PEG–Hb conjugates as well as EAF dextran PEG conjugates. This new platform certainly simplifies the generation of detxan Hb conjugates, besides this expanded approach of EA chemistry adds a new dimension to the concept of engineering plasma expander like properties of Hb for generating oxygen therapeutics, by combining PEG-Hb and dextran to come up with novel tertiary conjugates of polymers with novel plasma expander like properties that is not seen with either dextranylated Hbs, or the oligomeric conjugates of Hbs. In terms of structure, the dextran Hb conjugate may be considered as a necklace with glucose units as its individual beads and decorated with multiple copies of Hb bound tightly to some of the beads. The EA chemistry helps us to redesign and customize the linkage chemistry to modulate the structure Hbs conjugated to it as hanging pendants, and modulate the chemistry of attachment and the length of the EA to optimize the properties.

Accordingly, this structure of dextran Hb conjugates is very distinct from the PEG Hb conjugates discussed under Sect. 11.3.3. In PEG–Hb, Hb molecule is the central hard-core globular polymer from which multiple copies of long PEG chains are projecting out as molecular clouds. Accordingly, we should expect very different solution and hydrodynamic properties for EAF dextran PEG conjugates, and that the impact of these molecules on the flow properties and their interactions with the endothelial surface and/or RBCs may be significantly different when placed in the plasma.

Therefore, it will be intriguing to develop a new platform for generating dextran conjugates with Hb as a central core with multiple copies of smaller dextran chains projecting out from the central protein core in much in the same way as EAF PEGylated Hbs (see Sect. 11.3.3 for details). This will provide a unique opportunity for us to establish the relative advantages and the disadvantages of engineering plasma expander like properties by EAF PEGylation versus EAF

dextranylation in terms of designing oxygen carrying plasma expanders. Such an effort is currently underway at Einstein using monofunctionally-modified dextran, the functionalization dextran being at its reducing end.

#### 11.3.2.4 Conjugation of Hb to a Globular Synthetic Polymer

Synthetic polyfunctional polymers rather than elongated biopolymers like dextran or starch could also be used. Dendrimers represent one such class of molecules. Kluger and his colleagues (Hu and Kluger 2008; Gourianov and Kluger 2003) have developed a variety of intramolecularly crosslinked Hbs, particularly targeted to the $\beta\beta$-cleft of the molecule. A new approach for increasing the molecular size of Hb, i.e. a product with many copies of Hb tetramers been advanced by using dendrimers as the central core. In this approach the attempt is to surface decorate dendrimers with intramolecularly cross-linked Hbs. Kluger et al. used a trifunctional crosslinking reagent that introduces the desired intramolecular crosslink between the $\alpha\beta$ dimers of Hb within the $\beta\beta$-cleft and this engages two of the functional groups of the reagent in covalent linkage, and the third functional group of this trifunctional reagent is free to be used to attach the crosslinked Hb to a polyfunctional dendrimer. The chemistry of conjugation of the crosslinked Hb to the dendrimer is dictated both by the molecular excess of intramolecularly crosslinked Hb and the molecular dimensions of the dendrimer; this approach gives an excellent opportunity to generate size enhanced forms of intramolecularly crosslinked Hbs of desired molecular dimensions (Kluger and Zhang 2003).

### *11.3.3 Conjugation of Multiple Copies of PEG Chains to Hb: Surface Decoration of Hb with PEG Chains*

The concept and the technology of attaching PEG, an inert hydrophilic polymer of ethylene oxide, to proteins was first introduced by Davis in early 70 s and its usefulness as a drug delivery system has been extensively developed by Abuchowsky as the President of Enzon and now continuing as the President of Prolong Pharmaceuticals (Abuchowski and Davis 1979; Abuchowski et al. 1977). This technology is now referred to as "PEGnology". The covalent attachment of PEG chains to therapeutic protein was originally conceptualized to "mask" the antigenic epitopes of therapeutic protein from the host's immune system, i.e. to reduce the immunogenicity of the molecule and hence increase the circulation time. The PEGylation is also known to increase the molecular size of the protein, particularly much more efficiently than by attaching a protein of comparable molecular size. Accordingly PEGylation of protein is much more effective in increasing the molecular dimensions, i.e. the hydrodynamic volume (size in solution) of the protein and this approach also prolongs circulatory time. PEGylation also provides an increased solubility to protein therapeutics.

#### 11.3.3.1 Early Versions of PEG Conjugated Hbs

Many PEGylated forms of Hbs have been developed over the years. However two versions of PEG Hb that were developed by two pharmaceutical industries, Ajinomoto and Enzon, have impacted the field of blood substitutes resulting in a reemergence of the interest and optimism (Nho et al. 1994; Bradley et al. 1994). The studies of PEGylated Hbs along with the studies of prototype PEGylated albumin are expected to redefine the design of resuscitation fluids with and without oxygen carrying capacity, making a positive impact on the practice of transfusion medicine.

The Ajinomoto version of PEG-Hb uses a low oxygen affinity Hb derivative, the pyridoxylated Hb and the PEGylation is carried out using bifunctional PEG reagent, bis succinimidyl ester of PEG acid (Yabuki et al. 1990; Agishi et al. 1988). The bifunctional PEG reagent introduces both inter and intra molecular cross-linking into Hb as well as surface decoration of Hb, a monofunctional modification of pyridoxylated Hb. The product generated has a molecular mass of ~90,000, with an average modification of ten copies PEG-3000 on a molecule of Hb and has an oxygen affinity around 18–20 mm Hg. Enzon developed another version PEG–Hb using bovine Hb. In this approach bovine Hb was surface decorated using PEG-5K succinimidyl carbonate; this conjugation chemistry generates a urethane linkage between the amino groups of Hb and PEG chains. The Enzon version is a decaPEGylated bovine Hb, with an oxygen affinity around 12 mm Hg. This PEGylated Hb has a calculated molecular mass of ~115,000. The Ajinomoto PEG-Hb has both intra and intermolecular crosslinks, and had very little dissociable dimers. The Enzon PEG Bovine Hb has no intramolecular crosslink, and has no small molecular weight products (of the molecular size of $\alpha\beta$ dimers).

#### 11.3.3.2 Emerging New Concepts for Design of Blood Substitutes

In the mid 90 s Bob Winslow and his colleagues as well as Marcos Intaglietta and his associates, undertook a detailed comparison of the vasoactivity and microcirculatory aspects of these PEG Hbs, respectively, and compared these properties with those of $\alpha\alpha$-fumaryl Hb, the most hypertensive molecule. The Enzon PEGylated Hb was non-hypertensive, hence became an exception to rule that Hb and its derivatives are hypertensive. However, Enzon PEG Hb scavenges NO in vitro as efficiently as the other derivatives of Hb previously designed as blood substitutes. Thus the surface decoration of bovine Hb with ten copies of PEG 5K chains endows the molecule with some unique molecular properties that facilitates the attenuation of the in vivo hypertensive activity of bovine Hb.

The molecular properties of decaPEGylated bovine Hb are very distinct as compared to those of other Hb derivatives previously designed as blood substitutes. These properties are: (i) it is a high oxygen affinity Hb derivative, and (ii) it exhibits solution properties comparable to colloidal plasma expanders (hetastarch

or dextran 70). The plasma expanders like properties are not readily attainable with other Hb derivatives; these properties include (a) larger molecular volume as compared the material as compared to the to the molecular volume of a protein of comparable molecular mass (weight), (b) a viscosity higher than that of a protein solution at a comparable concentration, and (c) higher colloidal osmotic pressure as compared to the unconjugated protein of comparable concentration.

The Enzon PEGylated Hb has high oxygen affinity as compared to Hb and it is also nonhypertensive. Winslow and his colleagues, on recognition of this correlation advanced a new concept for the design of blood substitutes. They argued that earlier design strategies for blood substitutes focusing on species with low oxygen affinity comparable to that of Hb in RBCs are flawed (Vandegriff et al. 2003; McCarthy et al. 2001). They argued that low oxygen affinity of the blood substitutes designed earlier results in the unloading of the oxygen by these molecules in the arterial side of the circulation before reaching the capillaries leading to the activation of the autoregulatory principles. Accordingly, Winslow hypothesized that the oxygen affinity of Hb designed as blood substitutes should be higher than Hb and should not be comparable to that of RBCs. According to this new concept, which was supported by the physiological studies with hyperoxia, the hypertensive activity of the early designs of the blood substitutes have failed not just because they scavenge NO in situ, but because these products released oxygen prematurely at the artery and arteriolar level inducing vasoconstriction through auto-regulatory mechanisms. The observation that Ajinomoto PEGylated Hb has an oxygen affinity around 18 mm Hg is hypertensive even though it carries 10 copies of PEG 3K chains (total mass of 30 K) suggests that the oxygen affinity of Hb based oxygen carriers should be higher than Hb for the designed molecules to be nonhypertensive.

On the other hand, Intaglietta and his colleagues focused on the other unique aspect of the Enzon PEGylated Hb, namely its plasma expander like molecular and solution properties. They recognized, based on their extensive experience with plasma expanders with a wide range of viscosity, the improvements in the functional capillary density of animals transfused with Enzon PEG Hb, a phenomenon seen with many plasma expanders. Accordingly, they concluded that it is the plasma expander like properties of PEGylated bovine Hb that contributes to the attenuation of the vasoconstrictive activity of acellular Hb. The molecular and the solution properties of Enzon PEG Hb are the indeed properties of the resuscitation fluids. Accordingly, they advanced the concept that engineering plasma expander like properties to Hb as a novel strategy to design non-hypertensive Hbs for application as oxygen therapeutics.

#### 11.3.3.3 Extension Arm Facilitated (EAF) hexaPEGylation of Human Hb Using Mal PEG-5K Attenuates its in vivo Hypertensive Activity

The combination of these two new concepts discussed above implicate that increasing the hydrodynamic volume of Hb, viscosity and COP of Hb along with an increase in the oxygen affinity should be very effective approach to design nonhypertensive Hbs as oxygen therapeutics. Generation of new high as well as low oxygen affinity PEGylated Hbs with new PEGylation chemistry, with different PEGylation pattern (defined number and size of PEG chains) and with different levels of oxygen affinity is necessary to fully understand and delineate the molecular aspects of PEGylation induced attenuation of the hypertensive activity of acellular Hb. The attenuation of hypertensive activity of bovine Hb on decaPEGylation with PEG 5K (Prolong/Enzon product) and absence of this effect on decaPEGylation of pyridoxylated human Hb (Ajinomoto product) could be considered a consequence of the unique aspects of the structure/conformation of PEG Hb and the conjugation chemistry used.

As a prelude to understand this structure function correlation of the chemistry and the pattern of PEGylation of Hb with the attenuation of vasoconstrictive activity, a novel simplified PEGylation platform was developed by Acharya, Manjula and Smith (Acharya et al. 2000) at Einstein. This approach was originally referred to as the thiolation-mediated maleimide PEG chemistry based PEGylation of proteins. This approach has been expanded since then and is now referred to as Extension Arm Facilitated PEGylation of proteins, EAF PEGylation.

This new approach introduces new thiol groups on the surface amino groups of proteins using 2-iminothiolane. This is the cyclic form a bifunctional protein thiolating reagent $\delta$-mercapto butyrimidate chains, that can generate thiols as the targeted site for PEGylation using maleimide PEG. The 2-iminothiolane, by itself, does not carry free thiols groups and these are generated in situ only after the reaction of the reagent with the protein amino groups, and accordingly protein could be incubated with maleimide PEG in the presence of 2-iminothiolane without the danger of the bifunctional reagent consuming the PEG reagent (Cumber et al. 1985). Thus this approach introduces an extension arm between the protein amino groups and the functional group of the PEG chains. Accordingly, this approach is referred to as EAF PEGylation. A schematic representation of the cross section of EAF PEGylated Hb showing different regions of the PEG Hb molecule generated by EAF PEGylation platform and direct PEGylation is shown in Figs. 11.3 and 11.4.

#### 11.3.3.4 Flexibility of the Extension Arm Facilitated PEGylation Platform

The EAF conjugation chemistry is a very flexible platform for surface decoration of proteins with natural/synthetic polymers or with other desired small molecules

**Fig. 11.3** Schematic representation of the cross-section of the EAF PEGylated Hb showing the different layers of the molecule (not proportional to the actual thickness in the molecule) with differing packing densities. Center (*red*) is the high packing density protein core; in the case Hb this molecular radius of this region is 3 nm. The *yellow* layer represents a region of the extension arm, when this is engineered using 2-iminothiolane it about 1 nm. The thickness of this layer can be modulated by the selection of the heterobifunctional reagent for introducing the extension arms. The *green* region represents the covalent linkage with the spacer arm. The *yellow* and the *green* region are essentially spanning the protein hydration layer. The outer *blue* layer is the PEG shell and the PEGylation pattern; number and molecular size of the PEG chain dictate the thickness and packing density of this layer

(of therapeutic interest). In the initial platform developed the thiol groups were introduced at the distal end of the Extension Arms (EA) on the proteins as the targeted site to conjugate the desired molecules with a complementary functional group, with or without an extension arm. For the thiolation step of the EAF PEGylation, any heterobifunctional reagent carrying a protected thiol group as one of the functional group can be used in the EAF PEGylation reaction. In the original 2-iminothiolation mediated EAF platforms, the protection of the thiol is achieved intrinsically through the internal cyclisation of the bifunctional reagent, $\delta$-mercaptobutirimidate. Otherwise, the structure of bifunctional reagent reagents for EAF PEGylation can be represented by the formula X-(CH2)n-S–S-Py, wherein X represents the functional group that attaches the extension arms to protein, and these could activated carboxyl (succinimidyl or sulfosuccinimidyl ester or other active esters), or an imidate or an aldehyde group. The heterobifunctional reagents target the extension arms to the side chain amino functional groups of the protein and the active esters form an isopeptide linkage thereby neutralizing the original positive charge of amino function. Accordingly, this approach represents a

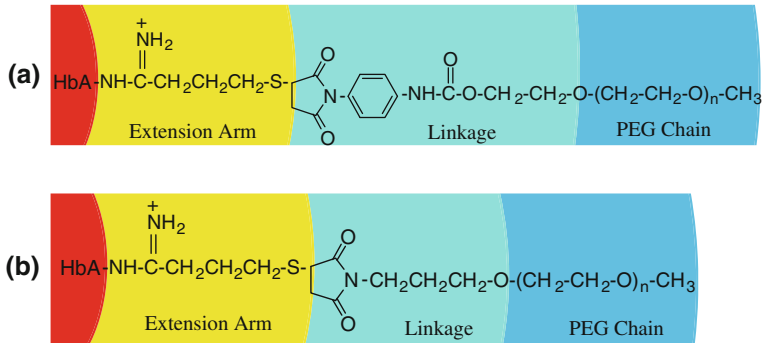


**Fig. 11.4** Exploded view of the structure of EAF PEG Hb exposing only the chemistry of the EAF-PEGylation. **a** Einstein EAF PEG Hb. **b** MP4 of Sangart. Only amino group of the Lys residues modified by extension arm chemistry is shown. The orientation of the side chains of Lys on the molecular surface of Hb, and hence the solvent accessibity of the amino groups will be very distinct. Accordingly, the thickness of the extension arm zone will be dictated by the oreientation of the Lys residue on the molecular surface. Protein-PEG linkage through the mediation of extension arm is depicted. The layer of spacer arm in the Einstein EAF P5K6 Hb is thicker than in the Sangart MP4, and the PEG-extension arm linkage in Einstein material exhibit higher rigidity than that in MP4 because of the presence of a phenyl ring in the former case

nonconservative version of EAF PEGylation. The heterobifunctional reagents with imidates and aldehydes (reductive alkylation) as amino reactive groups conserve the positive charge of the amino groups derivatized just in the original platform with 2-iminothiolane. These approaches are accordingly achieving conservative EAF PEGylation of proteins (Acharya and Manjula 2006). After the introduction of the EAs with the protection on the protein, the PEGylation of protein is achieved by incubating it with maleimide PEG in the presence of Tris (2-carboxyethyl) phosphine (TCEP). The latter is reducing agents that deprotects the thiols and generate the free thiol. Thus, the chemistry of the modification of the amino groups by EAs on the protein by the heterobifunctional group can be modulated to achieve either conservative or nonconservative EAF PEGylation of the protein (Acharya et al. 2011; Acharya and Manjula 2006).

The use of TCEP to deprotect the thiols at the distal end of the EAs with Hb is acceptable, as Hb does not contain any disulfide bonds. It should be noted that TCEP is an excellent reducing agent and can reduce the disulfide bonds of proteins (Miralles et al. 2013; Jones et al. 2012). Accordingly, it is recommended that when carrying out nonconservative EAF PEGylation protocol with disulfide-bonded proteins, for example albumin, milder deprotecting reagents like glutathione needs to be used to avoid the reduction of the intrinsic disulfide bonds of proteins.

### 11.3.3.5 Design, Synthesis and Characterization of EAF P5K6 Hb

A EAF hexaPEGylated human Hb was generated using this new EAF PEGylation platform, 2-iminothiolane as the choice bifunctional reagent to introduce the EAs and maleimido phenyl urethane PEG 5K as the PEGylation reagent. The structure of the PEGylated product generated by this conjugation chemistry is defined by the name EAF PxKy Hb, EAF refers to the conjugation chemistry, P here refers to the PEG chain of a given molecular mass, x defines the mass of the PEG used to generate the product in kilodaltons, K refers to the copies of the PEG-x chains conjugated onto the protein molecule, the exact number being defined by 'y'.

The extensively studied molecule generated by this new platform is EAF P5K6 Hb. In this EAF PEGylated Hb, both Cys-93($\beta$) of Hb are derivatized without the introduction of the EAs since the reaction was carried out under oxy conditions. The other four PEG chains are on the $\varepsilon$-amino groups of Hb conjugated through extension arm chemistry. Molecular model of one of the many isomeric forms of the EAF P5K6 Hb present in this sample is shown in Fig. 11.5. This hexaPEGylated Hb is a high oxygen affinity Hb; exhibited an oxygen affinity around 7–8 mm Hg in PBS buffer pH 7.4 and at 37 °C, the value for the unmodified Hb being 14 mm Hg. In bis tris buffers at pH 7.4 and 37 °C, its oxygen affinity is still around 7 mm Hg, the value for unmodified Hb being around 8 mm Hg. Thus EAF PEGylation of Hb with six copies of PEG 5K chains has limited influence on intrinsic oxygen affinity of the molecule. The high oxygen affinity is induced to the EAF PEGylated molecule in phosphate buffer. EAF PEGylation of Hb thus, significantly impacts the allosteric effector induced transition of the Hb to the low oxygen affinity form. This

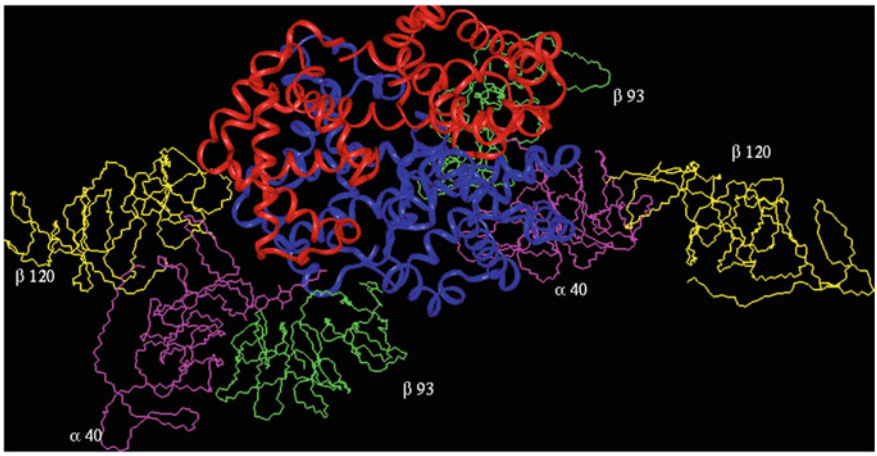


**Fig. 11.5** Molecular model of one of the isomeric forms of Einstein EAF HexaPEGylated Hb. EAF hexaPEGylated Hb refers to a population of species with an average number of six copies of PEG 5K per Hb molecule, and represents an ensemble of mutiple isomeric forms. The sites Lys-40($\alpha$), Cys-93($\beta$) and Lys-120($\beta$), the predominant sites of PEGylation in the EAF hexaPEGylated sample have been chosen here for molecular modeling studies of the hexaPEGylated species

makes the PEGylated Hb a high oxygen affinity Hb molecule under the physiological conditions. The EAF hexaPEGylation reduces the cooperativity only slightly, and the Bohr effect essentially completely lost.

The solution properties of the EAF hexaPEGylated molecule is most interesting aspect of this PEGylated Hb as these are the properties those make these molecules plasma expanders in vivo. A 4 gm% solution of EAF P5K6 Hb has a viscosity of 2.2 cp and COP of 65 mm Hg. The molecular radius of Hb is enhanced to 6 nm from its original value of 3 nm on PEGylation. The molecular radius of the EAF hexaPEGylated molecule is comparable to that of a polymer of intramolecularly crosslinked Hb with four copies of Hb tetramer. EAG hexaPEGylated Hb is conjugated with a total PEG mass of 30 K, and accordingly the molecular volume expansion protein seen PEGylation is nearly eight times as compared to that of comparable molecular mass of protein. This high oxygen affinity hexaPEGylated Hb was also nonhypertensive in experimental animal models. It is interesting to note that the new EAF P5K6 Hb is nonhypertensive (Manjula et al. 2005; Acharya et al. 2000), in spite of the fact it has a total conjugated PEG mass of only 30 K, and significantly lower than the total PEG mass conjugated present in Enzon PEG Hb. The total PEG mass in EAF P5K6 Hb is nearly equivalent to that in PEG Hb of Ajinomoto, but pattern of PEGylation is distinct and these two products have very distinct properties in terms of their in vivo hypertensive activity. Apparently, the pattern and chemistry of PEGylation appears to have a very significant influence on the PEGylation induced in vivo non-hypertensive activity of PEGylated samples of Hb.

#### 11.3.3.6 HexaPEGylation with PEG 5K but not Extension Arm Chemistry Contributes to the in vivo Non-hypertensive Activity of PEG Hbs

Another new high oxygen affinity hexaPEGylated Hb, TCP P5K6 Hb prepared by a direct PEGylation platform using isothiocyanato phenyl urethane of PEG 5K is also nonhypertensive (Acharya et al. 2011; Acharya and Manjula 2006). This PEGylation protocol does not introduce extension arms. The results suggest that extension arms do not contribute to the neutralization of the vasoactivity. Besides site selectivity of this direct hexaPEGylation, is very distinct as compared to EAF hexaPEGylation even though targeted to the surface amino groups. All the four amino terminus of Hb are quantitatively PEGylated in this case, and remaining two PEG chain are distributed on a number of $\varepsilon$-amino groups of Hb. Accordingly, it can be concluded that site selectivity of PEGylation is also not the determinant of the PEGylation induced in vivo neutralization of the vasoconstrictive activity of acellular Hb. Besides, we have hexaPEGylated Hb by direct PEGylation using reductive alkylation chemistry (Hu et al. 2005) and active ester chemistry (Li et al. 2008), and all the hexaPEGylation protocols induce the neutralization of the vasoactivity of Hb. Though all hexaPEGylated Hbs are high oxygen affinity Hbs, there were noticeable differences in the oxygen affinity of these molecules and also

in the COP, but that does not seem to have much correlation with the attenuation of natural vasoconstriction brought upon by the presence of intravascular acellular Hb.

#### 11.3.3.7 Extension Arm Chemistry Facilitates the Attenuation of PEGylation Induced Weakening of Interdimeric Interactions of Hb

Enzon decaPEGylated Hb is the first uncrosslinked derivatized Hb generated for application as a blood substitute. Nonetheless it does not induce nephrotoxicty, however this is not a chemical proof that the decaPEGylated Enzon bovine Hb does not dissociate and exists only as a PEGylated tetrameric species in vitro or in vivo. The molecular dimension of Enzon PEG-Hb molecule has been calculated to be about 15 nm based on its colligative properties (Vandegriff et al. 1997). However, this calculation makes an assumption that dodecaPEGylated bovine Hb exits in a tetrameric form and PEGylation has not induced a weakening of the interdimeric interactions of the tetramer. The molecular radius of Enzon PEG Hb is only around only 5.5 nm when determined by dynamic light scattering; this molecular radius of Enzon PEG Hb is nearly three times smaller than that calculated molecular radius, which based on the COP of the solution. COP of solution is colligative property and this suggests a weakening of the interdimeric interaction of bovine Hb by the succinmido carbmate mediated PEGylation platform used by Enzon. The PEG chain is linked Hb by a urethane linkage in this PEG bovine Hb.

A new hexaPEGlated human Hb with a thiouretane linkage has been prepared at Einstein. The molecular radius of this hexaPEGylated Hb generated by using isothiocyanato phenyl urethane of PEG 5K, i.e. TCP-P5K6 Hb is around 5.4 nm and this preparation is nearly 1 nm smaller than that of EAF P5K6 Hb. The molecular radius of the TCP P5K6 Hb increases to around 6 nm when an intramolecular crosslink (bis succinimidophenyl PEG 2K) is introduced between the two Cys-93($\beta$) of TCP P5K6 Hb and wherein the spacer arm (PEG 2K) is outside the central cavity (see Sect.11.3.1 for details). This increase in the molecular dimension is also accompanied with a concomitant reduction in the COP of the PEG Hb solution. These two aspects of the consequence of the presence of intramolecular crosslinking, reflects the presence of PEGylated dimers in the preparations of the hexaPEGylated Hb molecule in the absence of intramolecular crosslink. Besides, the molecular radius of TCP-P5K6-Hb($\beta\beta$ bisSP-P2) Hb is comparable to that of EAF P5K6 Hb. This reflects that the direct PEGylation results in a weakening of the interdimeric interactions of Hb tetramer and facilitates the dissociation of the hexaPEGylated tetramer to PEGylated dimers.

However, presence of intramolecularly crosslinks in Hb does not increase the molecular radius or reduce the COP of the EAF hexaPEGylated Hb. Thus while PEGylation induced plasma expander like properties of PEG-Hb facilitates the attenuation of vasoactivity of Hb, the extension arms of EAF P5K6-Hb attenuate the hexaPEGylation induced destabilization of the quaternary structure of Hb. The direct evidence for the role of EAs in attenuating the hexaPEGylation induced

weakening of Hb has been provided in the studies of Ananda and Acharya (Ananda et al. 2012).

The ability of extension arms to neutralize/attenuate the PEGylation induced destabilization of the quaternary structure is not absolute; it is a function of the extent of PEGylation. At the stage of the hexaPEGylation, there is very little, but still noticeable increase the tetramer-dimer equilibrium dissociation constant (Figs. 11.6 and 11.7). On the other hand at the stage of octaPEGylation of Hb, the dissociation constant increases to nearly 2 mM from 40 μM for hexaPEGylated Hb. At the stage of the tetraPEGylation of Hb with PEG 5K; the weakening of the interdimeric interactions is essentially absent.

We have also developed protocols for reversible protection of thiols of Cys-93($\beta$) during EAF PEGylation of oxy Hb (Acharya and Manjula 2007) so that EAF PEGylation is exclusively targeted to the ε-amino groups of the surface Lys residues of Hb when it is carried out under oxy conditions. The protection of the thiol of Cys-93($\beta$) is also accomplished, when the EAF PEGylation is carried out under deoxy conditions (Portöro et al. 2008). When the Cys-93($\beta$) is reversibly protected under the oxy conditions, stability of the tetrameric structure of resulting EAF P5K6 Hb, wherein all EAF PEGylation reactions are targeted to the ε-amino groups of Hb, is indistinguishable from the unmodified Hb (Acharya and Manjula 2007). It may be noted that the tetramer stability of EuroPEG Hb

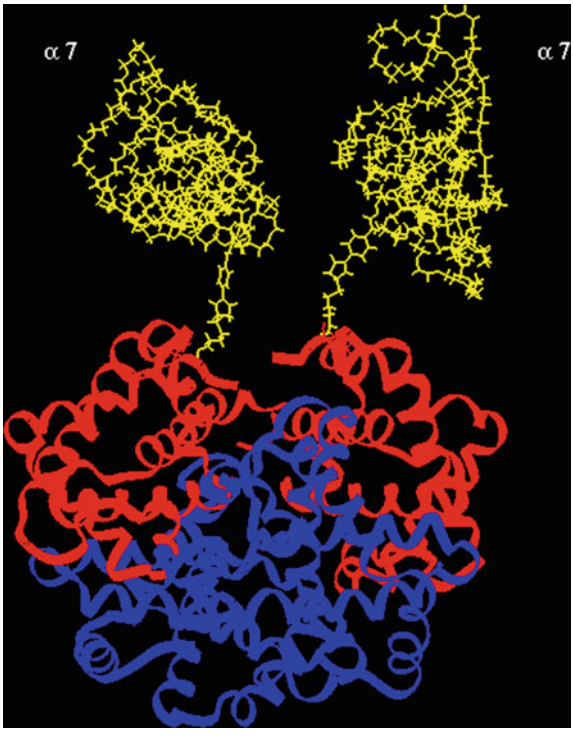


**Fig. 11.6** Molecular model of EAF diPEGylated Hb with the PEGylation targeted to the ε-amino group of Lys-7(α). The presence of extension arm between the amino group and the domain of the PEG chains minimizes any potential interactions between the molecular surface of Hb and the PEG-chains. This apparently, reduces the impact of the PEG chains on the weakening of the interdimeric interactions of the Hb tetramer as compared to the conjugation of PEG chains directly to the amino groups of the protein without the intervention of extension arms

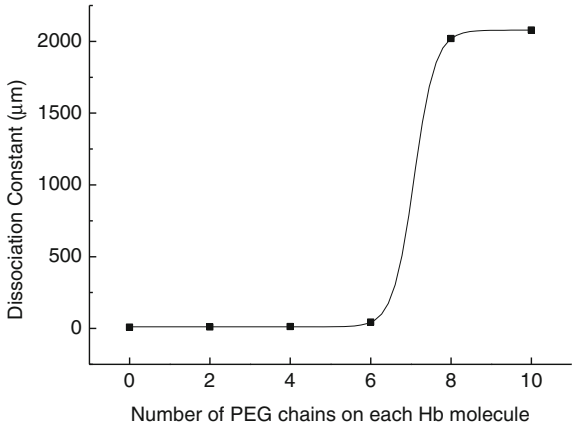


**Fig. 11.7** Influence of Pattern of EAF PEGylation used for surface decoration of Hb with PEG chains on the Dimer-Tetramer equilibrium. The dissociation constants were calculated by using size exclusion of the PEGylated samples. The position of αα-fumaryl Hb surface decorated using the same PEGylation pattern as the uncross-linked sample used for the position of the undissociated material. The EAF PEGylation beyond the stage of hexaPEGylation weakens the interdimeric interactions. Accordingly, it is better to keep the PEGylation below the hexaPEGylation level to minimize the formation PEGylated Hb dimers in vivo

(Portöro et al. 2008) has been reported to be higher than MP4 of Sangart, even though both have been generated using EAF PEGylation protocols. The primary difference between the two products is the site selectivity of PEGylation at Cys-93($\beta$). In MP4, the Cys-93($\beta$) is PEGylated while in EuroPEG Hb it is free.

#### 11.3.3.8 PEGylation Induces a Preferential Stabilization of the Oxy-Conformation of Hb

Extensive biochemical and biophysical analysis of Hb modified by NEM, and PEG maleimide modified Hbs (generated with and without the presence of 2-imnothiolane) have shown that PEGylation facilitates the stabilization of the oxy conformation of the molecule. This comes primarily from modification of Cys-93($\beta$) by maleimide. However, the PEG-shell of EAF P5K6 Hb also contributes to this stabilization of the oxy conformation of the tetramer (Khan et al. 2001). The phenyl linker between the maleimide moiety and PEG chains also appears to influence this structural/conformation properties of Hb, since the presence of an ethyl linker instead of the phenyl linker (Fig. 11.4) impacts these biophysical properties. The molecular modeling studies of this class of PEGylated Hbs have shown that the presence of phenyl urethane linkage between the maleimide moiety and the PEG chain versus the ethyl group, induces a degree of rigidity to the PEG chains, i.e., it reduces the dynamic flexibility of the PEG-chains.

#### 11.3.3.9 P5K2 Hb, P10K2-Hb and P5K4 Canine Hb are also Non-hypertensive

PEGylation of Hb appears to be an alternative approach to generate non-hypertensive Hbs to the direct approach of site directed mutagenesis that reduces intrinsic NO binding by Hb. The mapping of the relative significance of the PEGylation induced plasma expander like properties with the nonhypertensive activity of PEGylated Hbs, the correlation of the plasma expander like properties induced by PEGylation with the pattern, conjugation chemistry and extent (number) of PEGylation will facilitate optimization of the approach to attenuate hypertensive activity. EAF hexaPEGylation of Hb using PEG 5K was initially considered as adequate to essentially attenuate/neutralize the in vivo hypertensive activity of acellular Hb significantly and thereby provides the opportunity to take advantage of (harness) the therapeutic activity of Hb. As noted earlier, nonhypertensive Hbs with six copies of PEG 5 chains could be generated by direct PEGylation using acylation, thiocarbamoylation, and reductive alkylation chemistry based PEGylation platforms as well. However, a quantitative comparison of the PEG mass conjugated with the attenuation of hypertensive activity is not available as of now.

PEGylated Hbs with lower number of PEG K chains, for example Hb PEGylated directly with 2 copies of PEG 5K chains using maleimide PEG and canine Hb PEGylated directly with 4 copies of PEG-K chains exhibit a significant level attenuation of the hypertensive activity of Hb (Acharya et al. 2007). Similarly diPEGylated Hb generated maleimide PEG 10K as well as EAF hexaPEGylated Hb using maleimide PEG 3K and used at 6 % Hb is also non-hypertensive in extreme hemodilution studies in hamster. Thus, attenuation of the in vivo hypertensive activity of acellular Hb is not a correlate of chemistry or pattern of PEGylation, i.e. viscosity or COP of the solution of the PEGylated molecule.

#### 11.3.3.10 PEGylation Induced Attenuation of the Hypertensive Activity of αα-fumaryl Hb

The intramolecularly crosslinked Hb, αα-fumaryl Hb, is even more vasoactive than the other modified Hbs developed as blood substitutes. HexaPEGylation of αα fumaryl Hb EAF hexaPEGylation as well as by direct PEGylation platforms attenuates the in vivo hypertensive activity of the crosslinked Hb in the extreme hemodilution model. The higher in vitro hypertensive activity of this molecule did not neutralize the ability of PEGylation to attenuate the in vivo hypertensive activity of the molecule. DiPEGylation of αα fumaryl by direct PEGylation of the Cys-93($\beta$) using maleimide PEG 5K, tetraPEGylation of the molecule isothiocyanato phenyl urethane of PEG 5K and reductive hexaPEGylation of the molecule with six copies of PEG 2K propionaldehyde have been investigated. The resulting microcirculatory responses (in particular functional capillary density) show that the overall PEGylation induced attenuation of the in vivo hypertensive

activity of cellular Hb are not significantly impacted by the presence of αα-fumaryl crosslink in the molecule. However, different PEGylation chemistry and platform influences on the molecular and solution properties of the products in a PEGylation chemistry/platform dependent fashion. Thus the PEGylation induced non-hypertensive activity appears to have a significant buffering range to neutralize the hypertensive activity of Hb.

As noted earlier, the higher hypertensive activity of αα-fumaryl crosslink may be related to the lowered oxygen affinity of this molecule, and concomitant influence on the conformational aspect of the molecule. The structural conformational changes of Hb induced by αα-fumaryl crosslinking also extensively increases the rate of autoxidation as well as heme exchange reactions (Hu et al. 2008) but has very limited impact on the in vitro NO binding activity. Thus the very high in vivo hypertensive activity of this crosslinked Hb may also be related to the enhanced in vivo toxicity effects mediated through increased autoxidation, heme exchange and the generation of heme degradation products. The polynitroxylation mediated attenuating of the in vivo hypertensive activity of this crosslinked Hb (Buehler et al. 2000) has been attributed to the attenuation of the effects induced by activity of heme degradation products. The heme degradation products leads to the generation of free radicals and the enhanced hydrogen peroxide production in situ, this activity in the plasma decreases NO bioavailability in the system. Accordingly, the PEGylation and polynitroxylation of αα-fumaryl Hb attenuate the in vivo hypertensive activity of the molecule by mechanisms those are very distinct from one another and should be investigated in detail.

#### 11.3.3.11 Hemospan and Euro-PEG Hb

Extension arm chemistry (Cumber et al. 1985) designed at Einstein for inducing click chemistry concept and to the PEGylation reaction, as well as the hexaPEGylation pattern of PEGylation using maleimide PEG 5K to generate vasoinactive EAF P5K6 Hb (Acharya et al. 1998, 2005) was selected by Sangart. However, the hexa-PEGylated Hb developed by Sangart for commercialization as a blood substitute under the trade name Hemospan is not identical to EAF P5K6 Hb of Einstein in terms of the structure of the product. As noted before, Sangart believes and advocates the high oxygen affinity, low cooperativity and low Bohr effect of Hb derivatives as essential features of the molecules optimized for their application as blood substitutes. These are essentially the properties of myoglobin, an oxygen storage protein rather than an oxygen delivery molecule. Sangart believes that by designing the blood substitutes with these characteristic features, we will avoid the activation of autoregulatory principles by low oxygen affinity Hbs and the consequent vasoconstrictive activity (Vandegriff et al. 2003).

Though, Sangart decided to use extension arm chemistry for the preparation of their PEG Hb, it decided to replace the rigid phenyl linker of maleimido phenyl urethane PEG 5K reagent of Einstein by a more flexible propyl linker. The impact of the loss of rigidity afforded by phenyl ring to the PEG chains, and of the absence

of a hydrophilic urethane linkage between the linker moiety and the PEG-chains is expected to impact the chemical reactivity of maleimide functional group, and the structure/conformation of the PEG-shell. However this aspect has not been investigated in detail so far, but it has been shown that the deletion of the phenyl urethane moiety of the PEG maleimide reagent of Einstein (use of maleimido ethyl PEG 5K) alters the influences the PEG shell on the quaternary structure of Hb.

Besides, Sangart carries out EAF PEGylation of Hb under oxy conditions using the two-step EAF PEGylation platform. At Einstein, the two-step approach is reserved for the use of the nonconservative EAF PEGylation (Acharya et al. 2000; Cole and Vandegriff 2011) platform to macromolecules, where the bifunctional reagent used to introduce the extension arm has a protected thiol as a mixed disulfide. Accordingly the thiolation step is isolated from the PEG conjugation step. In the two step EAF PEGylation used by Sangart, the reaction of Hb with 2-iminothiolane is carried out at higher protein concentration in the absence of PEG reagent, thiolated Hb with multiple copies of extension arms has accumulated in the reaction mixture. And after the thiolation step, the samples are diluted with PEG reagent, and hence the PEGylation is carried out at a lower protein concentration. In the one step approach, practiced at Einstein to generate EAF P5K6 Hb modification of Hb by 2-iminothiolane is carried out in the presence of PEG maleimide, i.e., thiolated protein does not accumulate in the system and thiols generated in situ are immediately PEGylated. Accordingly the kinetics of the PEGylation of Hb is also expected to be very distinct in the one-step platform practiced at Einstein vs the two-step platform practiced by Sangart. Besides, the reactivity of Hb for thiolation (for the modification by 2-iminothiolane) in the one-step platform carried out in the presence of functionalization PEG is expected vs the thiolation of Hb in the absence of PEG reagent may be expected to be impacted in the reaction mixture during reaction and the PEG conjugated initially influence of the sequence of PEGylation. Thus site selectivity of EAF PEGylation not expected to be not identical in the products generated by one-step platform vs two-step platform.

Accordingly, the Einstein EAF P5K6 Hb should not be expected to be identical to Sangart hexaPEGylated Hb, MP4, in terms of the all-structural aspects and the chemical composition and functional properties. Consistent with this thinking, MP4 exhibits a slightly higher oxygen affinity and a lower cooperativity as compared to EAF P5K6 Hb of Einstein. Besides, the hexaPEGylated molecule of Sangart exhibits a higher molecular radius of around 10 nm (Olofsson et al. 2008) vs the 6.5 nm of the Einstein product (Manjula et al. 2005). Hemospan is generally referred to as Maleimide PEG modified Hb, MP, and it has been formulated as 4 gm% and 8 gm% (with respect to Hb content) solution, MP4 and MP8 respectively.

MP4 represents most extensively studied PEGylated Hb molecule that has completed all three phases of clinical trials (Olofsson et al. 2008; Vandegriff et al. 2008; Young et al. 2007; Keipert et al. 2008). However, the results of the phase III clinical trials were disappointing in that MP4 was not significantly superior to hetastarch, and worked essentially as a plasma volume expander. MP4 has been

designed for targeted delivery of oxygen to hypoxic regions, and this aspect was not addressed in the clinical trials. However, the most encouraging aspect of MP4 and of course the encouraging news to the blood substitute community is the absence of any toxicity when infused with an EAF hexaPEGylated Hb sample. Thus PEGylation of Hb appears to contribute not only to the attenuation of the in vivo hypertensive activity of acellular Hbs but also to the attenuation of the autoxidation dependent in vivo reactions that results in reduced bioavailability of NO and accompanying inflammatory reactions. The autoxidation mediated in vivo toxicity has dominated field. However almost all the potential blood substitutes studies earlier are all essentially unPEGylated Hb derivatives; this apparently reflects the unique structural aspects that is endowed to the Hb molecule by the PEG-shell, and as we learn more about these structural advantages we will be able to carry out more refinement of the structure/properties of EAF PEGylated Hb and design PEGylated molecules that can deliver oxygen better.

Euro PEG Hb (Portöro et al. 2008) is another prototype EAF PEGylated Hb and is generated under the deoxy conditions. Just as in Einstein approach, 2-iminothiolane mediated one-step extension arm chemistry is used for the generation of Euro PEG Hb as well, but maleimido propionalimido PEG 5K is used instead of maleimido phenyl urethane of PEG. In this reagent, the presence of a hydrophilic group in the spacer arm of the PEG reagent present in the Einstein PEG reagent is conserved. In the Einstein PEg reagent the hydrophilic group is a urethane linkage and in PEG reagent used for the preparation of Euro PEG Hb it is an isopeptide linkage. Besides, Euro PEG Hb appears to be a heptaPEGylated Hb molecule and the Cys-93($\beta$) is not PEGylated as the reaction is carried out under deoxy conditions. All the PEG chains are on the thiols groups of Extension Arms on the $\varepsilon$-Lys residues of Hb. The heptaPEGylation of Hb implies that the sample is apparently a mixture of hexa and octaPEGylated Hbs. This heptaPEGylated Euro PEG Hb is also a high oxygen affinity Hb in physiological buffer systems (in phosphate buffers) in spite of the fact Cys-93($\beta$) is free in Euro PEG.

This observation is consistent with the observation that EAF P5K6 rHb generated using the rHb[Cys-93($\beta$)- > Ala] is also an high oxygen affinity species (Li et al. 2007) reflecting the high oxygen affinity inducing activity of EAF PEGylation targeted to exclusively to the $\varepsilon$-amino groups. At Einstein, Hb has also been EAF PEGylated exclusively at $\varepsilon$-amino groups under oxy conditions achieved through the reversible protection of Cys-93($\beta$) during EAF PEGylation and this product is also high oxygen affinity species. The tetramer dimer-tetramer dissociation constant of these PEGylated molecules with Cys-93($\beta$) free is indistinguishable from that of unmodified Hb; the presence of PEGylation on Cys-93($\beta$) has only very small influence on the dissociation constant. On the other hand, a comparison of the stability of quaternary structure of MP4 versus Euro PEG Hb has suggested that in MP4 the quaternary structure of the molecule (Caccia et al. 2009) has been weakened leading to the presence of dissociated PEGylated species. This may be the influence of one-step versus two-step EAF PEGylation platform. The extent of PEGylation in a preparation of MP4 (Vandegriff et al. 2008) appears to be more than six copies per Hb molecule when recalculated from

the composition of PEGylated globin chains presented in a recent structural analysis of MP4, and the number of PEG chains appear to be closer to ten copies of PEG 5K chains per Hb tetramer. At this level of EAF PEGylation, extension arms do not buffer much the PEGylation induced dissociation of Hb tetramers beyond hexaPEGylation (see Fig. 11.7) (Vandegriff et al. 2008).

#### 11.3.3.12 Pattern of PEGylation of PEG Hb Dictates the Efficacy of the Tissue Oxygenation when PEG Hbs are of Comparable Oxygen Affinity

The optimal oxygen affinity of derivatives of Hb for its application as a blood substitute has been a subject of considerable interest in recent years. Winslow advanced the idea underpinning the design $\alpha\alpha$ fumaryl Hb as blood substitute is flawed. He advocated a new concept that high oxygen affinity, very low cooperativity and absence of Bohr effect as desirable properties of Hb derivative to serve as a blood substitute, since a low oxygen affinity product will deliver all its oxygen on the arterial side of circulation inducing vasoconstriction. The choice of Hb EAF hexaPEGylated with maleimide PEG by Sangart (Hemospan$^{TM}$) as a blood substitute is primarily guided by the high oxygen affinity of this material and this is apparently the first test case of new high oxygen affinity concept. The absence of any noticeable differences in terms of tissue oxygenation between hetastarch and MP4 in the first clinical trial of MP4 was a real disappointment. On the other hand, the observation in the clinical trial that MP4 functioned essentially as a plasma volume expander has stimulated the field for design of PEGylated materials as novel plasma expanders.

Di, tetra and hexaPEGylated derivatives of uncrosslinked Hbs have also been generated at Einstein using maleimide PEG 5K. All three PEGylated derivatives are high oxygen affinity molecular species since all have the Cys-93($\beta$) PEGylated and attenuate the in vivo hypertensive activity of the acellular Hb to a considerable degree (Li et al. 2006, 2007). The oxygen affinities of these PEGylated species are comparable. Nonetheless, tissue oxygenation seen with the PEGylated materials in extreme hemodilution models is a reverse correlate of the extent of PEGylation of Hb, i.e. amount of PEG conjugated to Hb. Thus, it is concluded that the amount of PEG conjugated to Hb impacts the delivery of oxygen by PEG Hbs, and EAF P5K6 Hb is very poor in delivering the oxygen. Thus, oxygen affinity of PEG-Hbs by itself is not the driving force for oxygen delivery, the structure of the PEG shell that is distinct in these molecules may have a role in dictating the efficacy of oxygen delivery. The maleimide PEG used in the preparation of these three PEG Hbs, i.e. maleimido phenyl urethane of PEG, has a rigid linker between the PEG chains and maleimide moiety, and exhibit higher cooperativity than MP4. Besides P10K2 Hb delivers oxygen better than P5K6 canine Hb, in spite of the fact both have nearly the same total amount of PEG conjugated and comparable viscosities. The results suggest the pattern of PEGylation may be the dictating factor that influences the tissue oxygenation by PEG Hbs with a given oxygen affinity. The

role of pattern of PEGylation is further confirmed by the fact that EAF P3K6 Hb gives a level of tissue oxygenation comparable to that by P5K2 Hb, nearly two to three times higher than with EAF P5K6 Hb. These results establish the role of both PEGylation pattern and the total mass of PEG conjugated to Hb tetramer in tissue oxygenation and underscores the need to understand and delineate the molecular aspects of tissue oxygenation by PEG Hbs.

As noted earlier, presence of αα-fumaryl crossbridge in Hb lowers the oxygen affinity and this also results in an increase in the hypertensive activity of Hb (Li et al. 2006). Nonetheless, PEGylation induced attenuation of the in vivo hypertensive activity of the acellular Hb is not impacted to any significant level by presence of αα-fumaryl crossbridge. Even though the introduction of αα-fumaryl crossbridge in the PEG Hb lowers the oxygen affinity of Hb considerably as compared to the respective PEGylated uncrosslinked Hb, the tissue oxygenation by PEG Hb is not improved by the presence of the intramolecular crosslinking. Apparent stabilization of the deoxy quaternary conformational feature of Hb by intramolecular crosslinking Hb appears to markedly reduce the ability of the PEGylated tetramer to deliver oxygen.

Influence of intra and intermolecular crosslinking on Hb has been investigated in the extreme hemodilution model. High oxygen affinity outside the central cavity crosslinked Hb is as good as or even better than the low oxygen affinity αα-fumaryl Hb, or very low oxygen affinity Biopure product in terms of tissue oxygenation. These studies raises some fundamental questions that need to be addressed in terms of correlation between the oxygen affinity and oxygen delivery by PEGylated Hbs. Investigation of these issues should be considered to be as the primary requirements, before we can undertake the clinical application of PEGylated Hbs as oxygen carrying plasma expanders (Table 11.1).

The early strategies for the design of blood substitute advocated the maintenance of the oxygen affinity of acellular Hb close to that of Hb in RBC (in the presence of DPG). This is based on the fact that the oxygen saturation curves of RBC reflect an oxygen affinity around 30 mm Hg. This oxygen affinity is consequence of the presence of DPG in RBC and the interaction of DPG with deoxy Hb. The binding of DPG to Hb is conformation specific. The deoxy Hb binds DPG with very high affinity, but the oxy Hb does not bind DPG. In the absence of the interaction of DPG, the oxygen affinity of acellular Hb is around 14 mm Hg. The oxygenation of deoxy Hb occurs in lungs, and in the lungs there is an abundance of oxygen. The oxygen tension in the environment of lungs is high and this is an open system as it is equilibrium with the outside air. This assures the complete oxygenation of Hb in RBC. The release of oxygen from oxy Hb in the circulation to be delivers to the tissues takes place in a closed system in terms of oxygen tension. Here the oxygen tension is low and the release oxygen from oxy Hb is essentially dictated by the oxygen tension in the surrounding environment. The primary role of DPG in the lung appears to be in ensuring an uninterrupted release of oxygen from oxy Hb. As oxy Hb releases its oxygen, the deoxy Hb formed binds to DPG and becomes a very low oxygen affinity species. This prevents the rebinding of the

**Table 11.1** Molecular and solution properties of MP4, EAF P5K6 Hb and Euro-PEG Hb

| | MP4 | EAF P5K6 Hb | Euro-PEG Hb |
|---|---|---|---|
| Extension Arm Chemistry | 2-iminothiolane | 2-iminothiolane | 2-iminothiolane |
| PEG Reagent | Maleimido propyl PEG 5K | Maleimido Phenyl urethane of PEG 5K | Maleimido propionyl PEG5K |
| PEGylation Protocol | Two step process, oxy | One step process, oxy | One step process, deoxy |
| Excess PEG reagent removal | Tangential Flow filtration | Tangential flow filtration | Tangential flow filtration |
| Hb sample used | Stroma free human Hb | Purified Hb | Q-Sepharose purified Hb |
| Number of copies of PEG chains | 6–8 | 6–8 | 6 |
| Molecular radius (nm) | 10 | 6.2 | |
| Increase in the molecular volume ($nm^3$) | 4049 | 860 | |
| Packing density of PEG in the PEG shell ($dal/nm^3$) | 8.6 | 40.7 | |
| P50 (mm Hg) | 4–6 | 7–8 | 6 |
| Hill coefficient | 1.2 | 1.9–2.1 | 1.6 |
| Viscosity of a 4 gm% solution (cP) at 37 °C? | 2.5 | 2.2 | |
| COP at 4gm% solution (mm Hg) | 50 | 65–75 | |

oxygen by deoxyHb in the tissues. Thus oxy Hb can release all its oxygen, depending on the need of the tissues (Table 11.2).

Thus, though αα-fumaryl Hb has been designed to mimic the oxygenation of deoxy Hb inside the RBC, it is expected to release its oxygen preferentially as compared to RBC since oxy Hb does not bind DPG and its oxygen affinity is considerably higher than that of αα-fumaryl Hb. Dexoy αα-fumaryl Hb does not bind DPG, there is no difference in the oxygen affinity of αα-fumaryl Hb in the presence vs in the absence of DPG (allosteric effectors).

Accordingly, we propose that the oxygen affinity of acellular Hb, not modulated by the reversible binding of allosteric effector, designed as oxygen therapeutic, should be in the mid-range between that of free Hb (14 mm Hg) and that of RBC (30 mm Hg). However, a P50 around 14 mm Hg for a doubly modified Hb, EAF hexaPEGylated αα fumary Hb, will also not equivalent of the oxygen affinity of unmodified Hb as the former represents an additive effect of the deoxy conformation stabilizing αα-fumaryl intramolecular crosslinking and oxy conformation stabilizing EAF hexaPEGylation.

**Table 11.2** Influence of pattern of EAF PEGylation for surface decoration of Hb on the dimer tetramer equilibrium

| Sample | Dissociation constant (μM)[a] |
|---|---|
| Control Hb | 8.5 |
| DiPEGylated Hb | 10.5 |
| TetraPEGylated Hb | 13.5 |
| HexaPEGylated Hb | 43.2 |
| OctaPEGylated Hb | 2019.2 |
| Deca-PEGylated Hb | 2078.2 |

[a] The dissociation constants were calculated by size exclusion of the PEGylated samples on Sepharose 12 columns. The position of αα-fumaryl Hb surface decorated using the same PEGylation pattern as the uncross-linked sample used for the position of the undissociated material

### 11.3.3.13 Novel Structural and Functional Aspects of EAF PEGylated Hbs

One of the important things to emerge from the extensive preclinical and clinical trials with Hemospan by Sangart is the low toxicity of this PEG Hb relative to the products studied earlier. The major in vivo toxicity of the blood substitute candidate, αα fumaryl Hb has been suggested to be due to release of heme degradation products from the met Hb generated in situ. The heme degradation products in the plasma from met Hb serve as the catalytic centers for the production of free radicals and of hydrogen peroxide. The toxic byproducts decrease the bioavailability of NO and initiate chains reactions inducing inflammation and ischemia. Conjugation of superoxide dismutase and catalase to Hb to generate Hb based oxygen carrier nanoparticles is an approach designed by Chang and his associates for overcoming the heme degradation product induced blood substitute toxicity (D'Agnillo and Chang 1993; Razack et al. 1997). The concept of engineering polynitroxylation (covalent attachment of "super oxide dismutase mimetic") into blood substitute was advanced by Synzyme to attenuate these complications. This approach indeed succeeded in overcoming the in vivo hypertensive activity of αα-fumaryl Hb (Buehler et al. 2000). However, polynitroxylation of Hb based oxygen carriers increase their rate of autoxidation, and the impact of this increased autoxidation has not been addressed in terms of the loss of oxygen carrying capacity of the oxygen therapeutics in situ and this has to be addressed in the future (Table 11.3).

(i) *Autoxidation and heme exchange reaction of PEG Hbs are attenuated significantly in vivo*:The lack of correlation between the in vitro rate of autoxidation and in vivo rate of autoxidation as well as very low toxicity, if any, with Sangart's MP4 as seen in the clinical trials represents the hallmark of this class of conjugated Hb. This is suggestive of the fact that the EAF PEGylation of Hb also facilitates in vivo the attenuation of the heme degradation product induced toxicity reactions of acellular Hb. PEG Hbs, both Sangart and Prolong Hbs are

**Table 11.3** Influence of molecular size of PEG chain in the EAF HexaPEGylation pattern on the packing density of PEG chain in the PEG shell

| Properties | EAF P3K6 Hb | EAF P5K6 Hb |
|---|---|---|
| Molecular radius (nm) | 4.84 | 6.12 |
| PEGylation induced Increase in radius (nm) | 1.74 | 3.02 |
| Packing density (D/nm$^3$) In the PEG in the shell | 51.43 | 39.4 |

The molecular radius of Hb is 3 nm; and is used in calculating the PEGylation increase in the molecular radius

used at a significantly lower concentrations of Hb (4 gm%) as compared to other unPEGylated blood substitutes (12 gm%). Accordingly, it may be argued that this has contributed to the absence or lower in vivo toxicity. Besides, with MP4 the rate of autoxidation in vivo is noticeably lower than in vitro. This may be also contributing to the diminished in vivo toxicity of MP4. The in vitro rate of autoxidation of diPEGylated Hb site specifically PEGylated at Cys-93($\beta$) as well as of EAF P5K6 Hb is significantly attenuated in the presence of plasma (Hu et al. 2008). Even more significant is that the rate of heme exchange of diPEGylated Hb is inversely correlated with the PEG chains mass used to generate the diPEGylated Hb. Future studies of PEG Hb should be focused on understanding this further and optimizing it to generate more stable versions of PEG Hbs (decreased rate of heme exchange).

(ii) *Vasodilatory activity of EAF PEG Hb*: EAF P5K6 Hb as well as MP 4 has been shown to have in vivo vasodilatory activity. Cabrales and Friedman (Cabrales et al. 2013) advanced the concept that PEGylation of Hb increases the intrinsic nitrite reductase activity of Hb and contributes to the vasodilator activity. EAF P5K6 Hb exhibits a higher nitrite reductase activity as compared to unmodified Hb (Lui and Kluger 2009; Cole and Vandegriff 2011). However the nitrite reductase activity is not a general characteristic induced by PEGylation, it is site specific effect of maleimide modification of the thiol group of Cys-93($\beta$) (Acharya et al. 2011). Accordingly, attempts to correlate nitrite reductase activity EAF PEG Hb to PEGylation as such are flawed.

(iii) *EAF P5K6 Hb and EAF P5K6 Albumin are novel resuscitation fluids exhibiting supra perfusionary activity*: Maleimide PEG modified Albumin (MPA) was generated by Sangart as non-oxygen carrying plasma expander control for MP4, an oxygen carrying plasma expander. MPA is generated using Baxter albumin, a transfusion material, using the extension arm facilitated PEGylation platform. Sangart has used the two-step EAF PEGylation platform for the surface decoration of human serum albumin, the same way Sangart has used earlier for Hb. Hb and Albumin, are very different proteins in spite of their similar molecular size ($\sim$66 kD). Hb is a four-subunit (tetrameric) protein, whereas Albumin is a single polypeptide chain protein. Hb has two reactive thiolsper mole [Cys-93($\beta$)] while human serum albumin has one free thiol coming from Cys-34; the human derived albumin has only

about 0.5–0.6 equivalents of reactive thiol, the lower amount of thiol represents the post translational modification of the residue. Hb has no disulfide bonds where as Albumin is a protein with 17 disulfide bonds. The stability of the disulfide bonds albumin in its thiolated form is also not clear. MPA has been reported to carry on an average five copies of PEG 5K chains. It may be noted that during the preparation of Baxter albumin, the human derived albumin is subjected to process of heating at high temperatures for ten hours (60 °C for 10 h). Almost all of the PEG chains in MPA are conjugated through extension arm chemistry. Molecular dimensions of this molecule have not been reported but this may be assumed to be close 10 nm in comparison to MP4. Both MPA and MP4 are vasodilatory. MPA does not have the heme center to generate NO in the plasma, through PEGylation induced nitrite reductase activity. Therefore, the hypothesis that PEGylation induced nitrite reductase activity to generate NO in situ, is the molecular mechanism could only be valid for Hb. Accordingly the molecular aspects of PEGylation induced supra perfusion seen with EAF PEG albumin has to be very distinct from the levels seen with EAF PEG Hb.

At Einstein, another version of PEG albumin, referred to as EAF P5K6 albumin has been generated using the on step extension arm facilitated PEGylation platform and using maleimido phenyl urethane of PEG 5K, the same protocol that that we had used to generate EAF P5K6 Hb. Besides, at Einstein we have used human derived albumin supplied by Sigma Aldrich and an albumin preparation that has not been subjected to the pasteurization process, heating at high temperature. Sigma Aldrich albumin has an average of about 0.6 mol of titratable thiol per mole of albumin, when subjected to EAF PEGylation this is PEGylated. The EAF P5K6 albumin carries around 6 copies of PEG 5K, and the molecular radius is around 7.5 nm, and is slightly larger than EAF P5K6 Hb. Accordingly, the composition and the structure of EAF P5K6 Albumin and of EAF P5K6 Hb generated at Einstein should be distinct molecular species as compared to MP4 and MPA, respectively, generated at Sangart, the only commonality between them being that the PEGylation in all these samples is accomplished by extension arm chemistry. Intaglietta and his colleagues have investigated potential clinical applications of EAF P5K6 albumin in experimental animal models and these studies are discussed in detail in a separate Chapter in this volume.

The 4 gm% solution of EAF P5K6 albumin is a low viscosity plasma expander and is nearly isoviscous with a 6 gm% solution of dextran 70. On the other hand 6 gm% solution of dextran 500 is a high viscosity plasma expander. The surprising and unique structural aspect of EAF P5K6 albumin is its ability to mimic, the physiological consequence seen with the infusion of high viscosity plasma expander, like dextran 500, i.e. vasodilation, and supra perfusion. Even though, a 6 gm% solution of dextran 70 is nearly isoviscous with EAF P5K6 albumin solution does not induce vasodilatation and supra perfusion. Consistent with this both solutions, EAF P5K6 Albumin and dextran 500, induce endothelial NO production.

The dextran 500 (6 gm% solution is used) has a hydrodynamic volume and viscosity that is nearly three times higher than that of EAF P5K6 (4 gm% solution used). Even though EAF P5K6 albumin is nearly isoviscous with dextran 70, and both have comparable hydrodynamic volume the latter does not induce vasodilation, supra perfusion or endothelial NO production. Accordingly, EAF P5K6 albumin and EAF P5K6 Hb have been classed as ***low viscosity NO producing plasma expanders*** with low viscosity, as the viscosity of this kind of materials is lower than that of blood.

Sriram et al. (2012) have recently shown that the common feature of all NO producing plasma expanders is their ability to enhance the shear thinning effect of RBC cells and thus contribute to the supra perfusion. The fundamental question remains what structural aspects of EAF PEG Hb and EAF PEG Albumin, the NO producing low viscosity plasma expanders, endows them with the ability to mimic the NO producing high viscosity plasma expanders (Acharya et al. 2011).

(iv) *Polynitroxylated EAF P5K6 Hb and Polynitroxylated EAF P5K6 Albumin*: As noted earlier, conjugation of an antioxidant molecule, Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl), to αα-fumaryl Hb neutralizes its in vivo hypertensive activity. This has been attributed to the attenuation of inflammation; the heme degradation product mediated pathological activity of the molecule in vivo. Antioxidant therapy of sickle cell disease (SCD) is one of the new strategies advanced to treat patients with SCD because the pathological consequences of the SCD appears to be resulting from the decreased NO bioavailability, a result of perturbed oxidative stress in vivo in SCD. Consistent with this, polynitroxylated albumin of Synzyme with 35 copies of conjugated tempol has been found to exert excellent therapeutic efficacy in attenuating the pathophysiology of SCD in transgenic sickle mouse (Kaul et al. 2006; Mahaseth et al. 2005). In view of the supra perfusionary aspects of EAF PEG albumin, this molecule was considered as a better "molecular truck" than albumin itself for the delivery of therapeutic agents. Accordingly, EAF P5K6 albumin tempol 12 was generated. The tempol molecules were also conjugated to EAF P5K6 albumin through the extension arm chemistry in contrast to the direct conjugation approach of Synzyme. The efficacy of both EAF P5K6 albumin and EAF P5K6 albumin Tempol-12 as therapeutic agent to treat SCD was assayed in two different models of SCD, NY1DD and S + S Antelles. The first model using NY1DD, the SCD disease is amplified by hypoxia/reoxygenation protocol before infusing the test materials. The results of the studies are summarized in Table 11.4. In the case of transgenic sickle mouse, S + S Antelles, the propensity of the test materials, EAF P5K6 Alb and EAF P5K6 Albumin T12 to improve the tissue oxygenation in the brain was investigated. Interestingly, both are excellent therapeutic agents for SCD.

**Table 11.4** Antioxidant therapeutic efficacy of EAF P5K6 Albumin vs its adducts with 12 copies of Tempol conjugated through EA chemistry in transgenic sickle mouse

| | Normoxia | | Hypoxia-Reoxygenation (NY1DD mice) | | |
|---|---|---|---|---|---|
| | Wild type (C57BL mice) | NY1DD mice | Untreated | PEG-Albumin (P5K6-Alb) | PEG-Alb-Tempol (P5K6-Alb-12T) |
| Diameter (μm) | 28.7 ± 1.3 (22)[a] | 28.4 ± 1.5 (15) | 28.4 ± 1.4 (14) | 27.6 ± 1.4 (11) | 28.6 ± 1.6 (13) |
| Red cell velocity (Vrbc) (mm/s) | 6.3 ± 0.6 | 2.5 ± 0.3[b] | 1.7 ± 0.2[c] | 5.0 ± 0.7[d] | 6.1 ± 0.7[d] |
| Wall shear rate ($s^{-1}$) | 1083 ± 86 | 449 ± 41[b] | 317 ± 37[c] | 988 ± 161[d] | 1062 ± 150[d] |
| Volumetric flow (Q) (nl/s) | 2.9 ± 0.48 | 1.1 ± 0.19[b] | 0.65 ± 0.08[c] | 1.7 ± 0.12[d] | 2.8 ± 0.64[d] |

Values are mean ±. [a] The number in the parenthesis represents the number of venules examined for hempdynamic parameters
[b] P<0.005−0.0001 versus normoxic wild type controls
[c] P < 0.05−0.023 vs respective normoxic valiues for NY1DD mouse, [d] P < 0.004−0.0001 vs untreated NY1DD mice subjected to hypoxia–reoxygenation

(v) *Anti inflammatory activity of EAF P5K6 Albumin*: A surprising and unexpected outcome from studies with SCD is the observation that EAF P5K6 albumin itself exhibited excellent antioxidant SCD therapeutic activity. It is well established that hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice (Kaul and Hebbel 2000). Thus the SCD therapeutic activity of EEF P5K6 Albumin seen in NY1DD mouse subjected to hypoxia/reoxygenation cycle is reflective of the anti-inflammatory activity of EAF P5K6. Is this a result of some intrinsic antioxidant activity of EAF P5K6 albumin molecule? We suggest that the NO producing activity of this low viscosity plasma expander discussed above, results in an increase in NO bioavailability. SCD has been characterized occurring as a consequence of decrease in the NO bioavailability. The ability of EAF PEG Albumin to induce the endothelial NO production presumably endows this molecule with an intrinsic anti-inflammatory activity. Alternatively, the EAF P5K6 Albumin has six thioether linkages, which can be readily converted to respective sulfoxides and regenerated back to sulfide. The antioxidant as well as the anti-inflammatory activity of sulfoxides is known well, accordingly the PEG conjugation chemistry introduced in our approach may have contributed to the observed anti-inflammatory activity.

EAF P5K6 Albumin is a prototype of EAF P5K6 Hb, accordingly it is expected that it will induce the same type of mechanisms for endothelial NO production enhancement; i.e. a low viscosity NO producing plasma expander mediated process when transfused into transgenic sickle mouse. It may be noted that the cyanomet form of EAF P5K6 Hb has been shown to exhibit vasodilation and supraperfusion just as EAF P5K6 Albumin, reflecting the need for the presence of heme for the active plasma expansion in vivo. MP4 has been used as a molecular vehicle to deliver carbon monoxide (CO) as an inflammatory reagent to regions of inflammation. Accordingly MP4CO has been advanced as a therapeutic agent for SCD and has given positive results in transgenic sickle mice. MP4CO is essentially non-oxygen carrying plasma expander till the CO is released in vivo. Accordingly further studies will be needed to map the relative contribution of CO of MP4 and of the active plasma expansion by the PEG shell of Hb in the attenuation of the pathophysiology of the sickle cell disease in transgenic mouse. It is conceivable that the PEG-protein conjugate induced endothelial NO production and modulation of NO bioavailability may be synergized with the CO of MP4CO. It may be noted that EAF P5K6 Albumin will not introduce any autoxidation and heme exchange mediated toxicity, and these expected with MP4CO, accordingly unless the CO makes major contribution towards the attenuation of papthophysiology of SCD, we prefer the use of EAF P5K6 Albumin for therapy of SCD.

Sanguinate of Prolong has also been used as carbonmonoxy adduct for the treatment of SCD. The potential role of endothelial NO production mediated anti-inflammatory activity in the SCD therapeutic activity has not been

addressed in the study of CO adducts of PEG Hb. Accordingly, it appears that therapeutic potential of these PEG Hb based therapeutic agents may be, at least in part come from the PEGylation induced endothelial NO production, and consequent vasodilation and supra perfusion.

(vi) *PEGylated Polynitroxylated Hb (PNPHb)*: Synzyme has used decaPEGylated bovine Hb as a molecular vehicle for the transport of Tempol moieties as antioxidant molecules (Hsia and Ma 2012). They have used their direct conjugation chemistry than the Einstein extension arm chemistry that gives a higher specific activity to the conjugated Tempol. The polynitroxylated decaPEGylated bovine Hb with about 15 copies of Tempol has shown therapeutic activity in SCD as well in traumatic brain injury models. Synzyme has demonstrated this activity earlier with their polynitroxylated albumin. Again the data comparing the therapeutic potentials of PEG-Hb and its polynitroxylated form, PNPH are not available at this time and represents an area that needs to be pursued. In our hands, EAF polynitroxylation of EAF P5K6 Hb has resulted in an increase in the rate of autoxidation of PEGylated Hb, a comparison of the relative merits and disadvantages of the PEG Hb derivatives vs EAF P56 Albumin is needed to identify the better therapeutic regent for therapy of SCD.

#### 11.3.3.14 Mutagenesis of His of Hb to Cys as Targeted Sites for Site Specific PEGylation: A Prelude to Generate Homogeneous PEGylated Hbs as Oxygen Therapeutics

EAF P5K6 Hb elutes as a reasonably homogeneous peak on the size exclusion chromatography on Superose 12 columns, with a small shoulder on the ascending side of peak. The major peak isolated by size exclusion chromatography, exhibits a very symmetrical elution pattern on analytical rechromatography. The symmetry of elution pattern of EAF P5K6 Hb and of that of MP4 represents only the overall homogeneity of the sample in terms of the hydrodynamic volume and not the chemical homogeneity of the samples with respect to the sites of PEGylation on the Hb molecule. At the high concentration of Hb and the limited amount of the thiolating reagent used in the preparation of these PEGylated Hbs, the thiolation is not a very site-specific event. Accordingly, the EAF hexaPEGylation just as other direct PEGylation platforms lacks the chemical homogeneity and hexaPEGylation represents an average substitution by PEG chains (Table 11.5).

Targeted PEGylation of the thiol group of Cys-93($\beta$) of oxy human Hb with maleimidophenyl urethane PEG has been used to generate site specifically diPEGylated Hbs. Maleimide PEG of different molecular sizes were used to generate P3K2 Hb, P5K2 Hb, P10K2 Hb and P20K2 Hb. This direct site specific PEGylation has also been associated with a significant level of attenuation of in vivo vasoconstrictive activity of Hb (Manjula et al. 2003). This approach of site specific

**Table 11.5** Solvent accessibility of cys residues in Hb introduced by directed mutagenesis

| Residue number | Accessible surface area (Sulfur) | Accessible surface area (Total) |
|---|---|---|
| B 93 CYS | 6.16 | 8.75 |
| D 93 CYS | 6.26 | 8.05 |
| A 20 CYS | 1.22 | 23.38 |
| C 20 CYS | 3.53 | 30.92 |
| A 112 CYS | 20.5 | 42.2 |
| C 112 CYS | 19.2 | 39.5 |
| B 117 CYS | 28.3 | 62.2 |
| D 117 CYS | 26.5 | 64.88 |

PEGylation of reactive and accessible intrinsic thiol groups of Hb using maleimide PEG has been extended to generate chemically homogeneous PEG Hbs with multiple copies of PEG 5K chains by using animal Hbs that carry additional reactive thiol groups. A number of animal Hbs that contain additional reactive surface Cys-residues, besides Cys-93($\beta$) have provided a new source of Hbs for generating chemically homogeneous PEGylated Hbs. P5K4 canine Hb and P5K6 feline Hb have been generated by this approach and these PEGylated Hbs also exhibited a considerable degree of neutralization of their hypertensive activity.

Chien Ho and his colleagues at Carnegie Mellon University have taken this concept of developing chemically homogeneous PEGylated Hb one step further. They reasoned that mutating one or more of the surface histidine residues of human Hb to Cys residues in a site specific fashion as the targeted sites for maleimide PEG based PEGylation will be the final and the desired approach of customized semi-synthesis of the desired PEGylated Hbs. In this novel approach mutant Hbs are produced through site directed mutagenesis with reduced NO scavenging activity, reduced in vivo autoxidation and/or hydrogen peroxide mediated autoxidation, and desired oxygen affinity. A desired level of PEGylation is engineered into such molecules thorough site directed mutation of surface His to Cys and conjugation of PEG chains to them either by direct or by EA chemistry based PEGylation. As a first step to realization of such a goal, three surface His residues have been identified for mutation into Cys as the targeted sites for PEGylation. The identified sites to introduce new surface Cys residues are His-20($\alpha$), His-112($\alpha$) and His 117($\beta$) and these mutants have been expressed and characterized. The location of these sites on the Hb molecule is depicted in Fig. 11.8.

The solvent accessibility of the thiol group of the newly engineered Cys residues into Hb is high, in particular, as compared to intrinsic thiol of the Cys-93($\beta$) of wild type, the most reactive Cys residue of Hb under oxy conditions. The tetraPEGylated recombinant Hb, with succinimidophenyl PEG 5K as well as succinimidophenyl PEG 3K chains conjugated directly at Cys-93($\beta$) and Cys-20($\alpha$) and also at Cys-93($\beta$) and Cys-117($\alpha$) have been generated and characterized. The oxygen affinities of these tetraPEGylated derivatives were around 7 mm Hg in PBS buffer pH 7.4 and are comparable to that of EAF P5K6 Hb. The

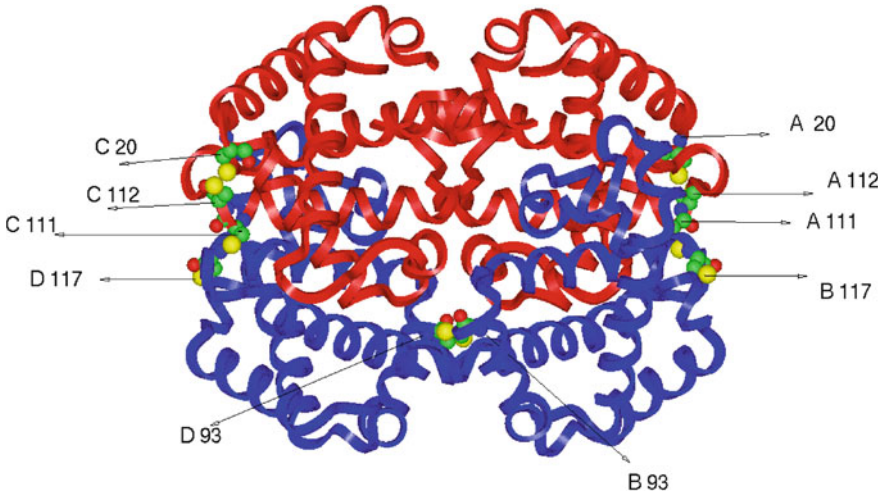


**Fig. 11.8** The model of human Hb depicting the Cys residues introduced in the Hb model. The three mutated residues are shown in the ribbon model of hemoglobin (4HHB) along with Cys B93, D93. The position of Cys-111(α) is also shown, as this corresponds to the position of canine Hb, that was used to generate the first hemogenous prepartions of TetraPEGylated Hb. The atoms are color-coded and sulfur atoms are shown in *yellow*. As discussed in the text, His is the normal resides at the sites indicated [except for Cys-93(*β*) which is residue present at this site in the wild type] have been mutated by site directed mutagenesis. All Cys residues introduced exhibit a higher solvent accessibility as compared to Cys-93(*β*). It may also be noted that all new Cys residues are located away from the *αα* end and the *ββ*-end of the central cavity of Hb. It may also be noted that when the mutant Hbs are tetraPEGylated, Hb with mutation of Cys-117(*β*) generates an assymmerically PEGylated molecule with all PEG chains on the *β*-chains

tetraPEGylated species with four copies of PEG 5K chains exhibited plasma expander like properties comparable to those of tetraPEGylated canine Hb. In the extreme hemodilution models all tetraPEGylated recombinant Hbs exhibited a mean arterial pressure comparable to canine tetraPEGylated Hb. However, only the PEGylated forms of His-20(α)-Cys recombinant Hb gave a level of FCD comparable to that of canine Hb.

Recombinant Hb[H117(*β*)C] has also been tetraPEGylated with PEG 10K on Cys93(*β*) as well as with PEG 5K. The microcirculatory properties of hamster infused with these homogeneous tetra PEGylated materials with a total 40 K PEG mass and with a total 20K PEG mass per mole were good. However excellent level of tissue oxygenation was seen only with Cys-20(α) mutant. The level of tissue oxygenation with this mutant Hb is close to the level of P5K2 Hb and P10K2 Hb and better than that with canine Hb. But tetraPEGylated mutant Hbs with PEG 3K at the same position elicited a very low response for FCD, but comparable levels of MAP and low to that of the molecule with 5K, and lower level of tissue oxygenation as compared to tetraPEGylated His-20(α)->Cys recombinant Hb. Accordingly, we conclude that His-20(α)–>Cys mutation is the best site for

generating mutant Hb for maleimide PEG 5 K targeted PEGylation of Hb in terms tissues oxygenation in extreme hemodilution models.

An interesting structural aspect of rHbs with these H to C mutations is that the reactivity of new Cys introduce to form adducts with maleimide PEG 5K in oxy form is higher than that of Cys-93($\beta$) oxy Hb. Accordingly, we can design conditions for site specific PEGylation of newly engineered Cys residue of mutant Hbs even in the presence of Cys-93($\beta$). However mutation of Cys-93($\beta$) to Ala, a mutant Hb discussed earlier, is preferred if a site selective diPEGylated Hb with maleimide PEG 10 K on Cys-20($\alpha$) turns out to be the desired site specifically PEGylated molecule as an oxygen carrying plasma expander.

As noted above tetraPEGylation of recombinant Hb($\alpha$H20C) with PEG 5 K increases the oxygen affinity to levels comparable to that of tetraPEGylated canine Hb, and EAF hexaPEGylated human Hb. The molecular radius of the tetraPEGylated Hbs is smaller by nearly by 1 nm, and the viscosity and COP is slightly lower than that of hexaPEGylated Hbs. Accordingly, the results of all the tetraPEGylated recombinant Hbs investigated, the question arises as to whether there is a degree of site selectivity in terms of improving the FCD and tissue oxygenation. The mutation of His-20($\alpha$) to Cys appears to be unique in this respect, with tissue FCD and tissue oxygenation levels only slightly lower than diPEGylated Hbs with PEG on Cys-93($\beta$). Given the known influence of PEGylation of Cys-93($\beta$) on the oxygen affinity of Hb, it is conceivable that site specific diPEGylation of Hb on Cys-20($\alpha$) in the r-Hb will be a preferred molecule as an oxygen therapeutic, and using such DiPEGylated r-Hb as a 6 gm% solution to improve the solution properties will be preferred design blueprint.

The results with rHbs have exposed a surprisng result, a nonequivalence of the PEGylation sites in terms of their ability for modulating the tissue oxygenation while essentially sites are nearly equivalent in terms of inducing the plasma expander like properties to Hb. Accordingly, EAF PEGylation of Hb will have to be refined further by developing new chromatographic platforms to facilitate the isolation of chemically homogeneous species PEGylated Hbs and the isomeric forms PEGylated Hbs with defined number of PEG chains. Identification of PEGylation sites that endow the PEGylated Hb molecule with better tissue oxygenation ability should result from these new investigations. In the absence of such approaches, site directed mutagenesis would be the only approach available to move ahead in this direction of designing novel oxygen therapeutics.

An oxygen affinity of 15 mm Hg is the preferred oxygen affinity for Hb based oxygen carrier (Acharya et al. 2007). The diPEGylated recombinant Hb, designed as discussed above will still have high oxygen affinity. Engineering additional low oxygen affinity inducing mutation(s) into this Cys-93($\beta$)->Ala and His 20($\alpha$) to Cys can be undertaken to generate diPEGylated Hb with lower oxygen affinity as compared to the diPEGylated Cys-20($\alpha$) mutant Hb. Presbyterian mutation, Asn-108($\beta$)->Lys is an excellent mutation to be considered towards developing low oxygen affinity diPEGylated Hb.

Since EAF PEGylation appears to have unique advantage of minimizing the impact of the PEG chain on the structure and function of Hb, it may be still be

advantageous to attach the PEG chains to recombinant Hbs with newly engineered surface Cys-20(α) and other lower oxygen affinity inducing mutations engineered into Hb. The EA chemistry has to be changed here slightly; monofunctional modification of the Cys mutant Hb will be carried out under deoxy conditions with a short bis maleimide and PEGylation will be achieved using thioPEG at the second maleimide moiety of in the monofunctionally modified Hb. Alternatively, given the high solvent accessibility of the thiol of Cys-20(α) of recombinant Hb, the extension arm can be placed on the functionalized PEG. For example, PEG propylamine could be first modified with sulfo EMCS [*N*-(ε-maleimidocaproyloxy)sulfosuccinimide ester], to generate a new maleimide functionalized PEG with extension arm (maleimido caproic acid) and this new functionalized PEG is used to PEGylate the recombinant Hb using maleimide based conjugation chemistry.

We have also been evaluating bovine Hb as the starting Hb molecule for generating lower oxygen affinity EAF diPEGylated Hb with diPEGylation with PEG 10 K exclusively targeted to ε-amino group of bovine Hb by carrying out the 2-iminothiolane based EAF PEGylation under deoxy conditions. A diPEGylated bovine Hb with an oxygen affinity around 16 mm Hg (control value for bovine Hb around 25 mm Hg) has now been generated. Therefore, alternatively, bovine Hb is also a potential molecule for site directed mutagenesis of its Cys-92(β) to Ala and of His-20(α) to Cys. Such a molecule is expected to be an excellent Hb candidate for generating a diPEGylated Hb (using maleimide PEG-10 or sulfo EMCS modified PEG 10 K propylamine) as novel oxygen therapeutic.

Thus, combining structure-based site directed mutagenesis of Hb and EAF PEGylation provides us with a powerful new platform with an unprecedented strength and opportunity to develop oxygen therapeutics, in particular to generate chemically homogeneous PEGylated Hbs. EAF PEGylation allows us to engineer resuscitation fluid properties and supra perfusion. EA chemistry helps us to mitigate the impact of PEGylation on the quaternary structure of Hb and subsequent pathophysiologcal consequences. Because of the higher stability of interdimeric interactions of some animal Hbs and lower intrinsic oxygen affinity (without allosteric effectors) relative to human Hb, some of these animal Hbs are expected to serve as a better natural low oxygen affinity Hb to engineer and to customize the molecular properties by site directed mutagenesis.

## 11.4 Conclusions

Enhancing the molecular size/dimension of Hb through conjugation was initiated as a structural strategy to attenuate the NO scavenging mediated hypertensive activity of acellular Hb. These classes of conjugated Hbs that achieve the goal have been discussed here. The design of conjugated Hb by oligomerization generates products with some attenuation of the in vivo hypertensive activity. Polymerization of Hb to molecular dimensions of 25 nm, that also increases the viscosity of the solutions of these polymers significantly relative of unmodified

Hb, results in a noticeable level of attenuation of hypertensive activity of Hb. Dextran Hb conjugates, wherein multiple copies of Hb are conjugated to dextran, an intrinsically viscous material used as a plasma volume expander, also neutralizes the vasoconstrictive activity of Hb. Conjugation of multiple copies of short PEG chains to Hb, PEGylation, also facilitates the neutralization of the hypertensive activity of Hb and this is accompanied by increase in hydrodynamic volume, viscosity and colloidal osmotic pressure. The molecular volume increase is minimum in PEGylation as to the very large increase needed in oligomerization for the neutralization of the hypertensive activity of Hb. Engineering extension arms between the PEG chains and the central protein core reduces the impact of PEG shell on the inter dimeric interactions of Hb and on the quaternary structure of Hb. Optimizing the number of PEG-5 K chains conjugated to four to six copies per molecule is critical also to preserve the "native-like" structural integrity of Hb molecule. But maintaining a level of structural flexibility to the Hb core of PEG-Hb to facilitate a better delivery of oxygen still remains a challenge. Extension Arm Facilitated PEGylation that induces supra perfusion is perceived by us as one of the strong and promising platform to tame the in vivo hypertensive activity of Hb. In view of the nonequivalence of the PEGylation sites of Hb to facilitate the tissue oxygenation, while the PEGylation sites appear to be equivalent in terms of inducing plasma expander like properties to Hb, a marriage of EAF PEGylation platform with site directed mutagenesis is suggested as the future avenue to achieve customized taming of molecular properties of Hb on one side and achieve the optimization of tissue oxygenation. This approach will involve new concepts in EA chemistry based PEGylation that is couple to new chromatographic platforms and these are developed and pursued by this team to lead us to new dimension as we travel to design and develop safer and efficacious oxygen therapeutics.

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# Chapter 12
# Cellular-Type Hemoglobin-Based Oxygen Carriers to Mimic the Red Blood Cell Structure

**Hiromi Sakai**

**Abbreviations**

| | |
|---|---|
| Hb | Hemoglobin |
| HBOCs | Hb-based oxygen carriers |
| RBC | Red blood cell |
| HbV | Hb-vesicles |
| LEH | Liposome-encapsulated Hb |
| HbCO | Carbonylhemoglobin |

## 12.1 Chemically Modified Cell-Free Hb and Encapsulated Hb

The concentration of hemoglobin (Hb) in healthy human blood is around 12–15 g/dL, making Hb the most abundant protein in blood. Hb is an oxygen binding protein that is compartmentalized in red blood cells (RBCs) with an intracellular Hb concentration of about 35 g/dL. Packed RBCs derived from blood donation can be stored only for 6 weeks in the US and for 3 weeks in Japan. Historically, a crude Hb solution was tested as a substitute for RBCs in (Von Stark 1898), but it was not successful because of various side effects. Since the late 1960s, chemically modified Hb solutions have been developed (Vandegriff and Winslow 1991). Many materials have progressed to use in clinical studies, but many such studies have been suspended because of side effects (Natanson et al. 2008).

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Recombinant human Hb was also tested, but it failed in clinical trials (Murray et al. 1995). Actually, an earthworm, as a lower organism, has no RBCs, but it does have gigantic Hb molecules. Mammalians, as higher animals, have RBCs for several physiological reasons. It seems difficult to create an RBC substitute with cell-free Hb solutions. Even though Hb is the most abundant protein in blood, it becomes toxic once released from RBCs.

We believe in the physiological importance of the cellular structure of RBCs, and continue to develop Hb-vesicles (HbV) as a cellular-type HBOC (Sakai et al. 2008a; Tsuchida et al. 2009). By considering the physiological importance of RBCs, it is easy to understand the side effects of cell-free HBOCs. An RBC has a biconcave disk structure with 8 μm long-axis diameter, encapsulating about two million Hb molecules (Mw. 64500) at a concentration of about 35 g/dL. The physiological reasons for Hb compartmentalization in RBCs are the following: (i) shielding direct contact of toxic Hb and vasculature (Burhop et al. 2004); (ii) prevention of extravasation of dissociated Hb dimers through renal glomeruli, and prolonged circulation time; (iii) circumvention of high colloid osmotic pressure and viscosity of concentrated Hb solution (Sakai et al. 2000); (iv) co-encapsulation of electrolytes, ATP, glycolytic, and metHb reducing enzymatic systems, etc.; (v) retarded reaction of Hb with NO and CO as vasorelaxation factors and retarded $O_2$-release in the vasculature (Sakai et al. 2008, 2010); (vi) RBCs tend to flow near the centerline in vasculature (centralization), avoiding contact with vascular walls where shear stress is the greatest. This flow style is appropriate for preventing hemolysis (Sakai et al. 2009); (vii) Moreover, the high viscosity of blood is mainly attributable to the presence of RBCs, producing a non-newtonian fluid, which is important for blood circulation, especially in microcirculation, from a physiological perspective.

## 12.2 Attempts to Produce Cellular Type HBOCs Using Polymeric Materials

Chang (McGill University) was the first to test encapsulation of Hb solution with a polymer membrane in 1957 (Chang 2007) as one example of “artificial cells”. In Japan, Kimoto and his colleagues tested Hb encapsulation from around 1961 using polystyrene, gelatin, and rubber membranes (Toyoda 1966; Kimoto et al. 1968; Kitajima et al. 1970). Although their attempts were original, they were unsuccessful: the particle size could not be reduced to less than capillary diameter (<4 μm). Later, polymeric materials of various kinds with biodegradable properties became available through the use of polypeptides (Arakawa et al. 1975; Palath et al. 2007), polycaprolactone, and polylactide (Zhao et al. 2007; Zhang et al. 2008) with much smaller diameters. These capsules have permeability of small ionic molecules, which would be advantageous for the reduction of intracellular methemoglobin by reducing agent dissolved in plasma. However, it is speculated that hydrolysis of the polymeric materials during preservation (before

injection) and during blood circulation might induce hemolysis: leakage of the encapsulated Hb. Polymersomes are new materials for encapsulation of Hb solution (Rameez et al. 2008). Kishimura et al. (2007) reported encapsulated myoglobin using PEGylated polyion complex vesicles (Table 12.1). These new materials have been mostly described in reports published in chemistry journals. They await detailed in vivo and in vitro examination to assess their safety and efficacy.

## 12.3 Cellular Type HBOCs Using Liposome

Bangham and Horne (1964) discovered the formation of vesicles (liposomes) when phospholipid was dispersed in aqueous phase. After this discovery, many researchers tested encapsulation of functional molecules in liposomes, especially for anticancer therapy. Djorjevici and Miller (1977) (University of Illinois, Chicago) reported encapsulation of Hb in liposomes, called "synthetic erythrocytes" (Table 12.2). Subsequently, many groups throughout the world attempted so-called liposome encapsulated Hb (LEH). However, most of those efforts were not successful because of their low encapsulation efficiency, polydispersibility of particle size, and instability. The US Naval Research Laboratory aggressively

**Table 12.1** Encapsulated Hb using polymeric membrane, and polymer-embedded Hbs

| Authors | Characteristics |
|---|---|
| Chang 2007 | First attempt of encapsulated Hb using polymer membrane |
| Toyoda 1966<br>Kimoto et al. 1968 | Encapsulated Hb using polystyrene, gelatin, rubber membranes |
| Arakawa et al. 1975 | Encapsulation with poly(lysine membrane) |
| Cedrati et al. 1994 | W/O emulsion using polylactide |
| Meng et al. 2003 | Methoxypolyoxyethylene-polylactide microcapsules |
| Baumler et al. 2005 | Polyelectrolyte microcapsules made with RBC template |
| Patton and Palmer 2006 | Hb-poly(acrylamide) hydrogel |
| Zhao et al. 2007 | Encapsulated with biodegradable polymers of PCL-PEG. |
| Palath et al. 2007 | Encapsulated with polypeptide multilayer nanofilms (PLGA and PLL) using $CaCO_3$ particle template |
| Kishimura et al. 2007 | PEGylated polyion complex vesicle encapsulating Mb |
| Rameez et al. 2008 | Biocompatible and biodegradable polymersome encapsulated Hb |
| Zhang et al. 2008 | Hb-loaded nanoparticles with PEG-PLP-PEG block copolymer |
| Shi et al. 2009 | Hb-conjugated micelles based on triblock biodegradable polymers |
| Chauvierre et al. 2010 | Hb is embedded on heparin coated poly(alkylcyanoacrylate) nanoparticles |
| Gao et al. 2011 | Cationic amylose-encapsulated bovine Hb |
| Duan et al. 2012 | Enclosing Hbs in $CaCO_3$ microparticles and modification with PEG. |

**Table 12.2** Trials of liposome encapsulated Hb

| Authors | Lipid composition | Characteristic preparation methods |
|---|---|---|
| Djordjevich and Ivankovich 1988 (first reported in 1977) | L-α-phosphatidylcholine/cholesterol/palmitic acid | Sonication |
| Gaber et al. 1983 | EYPC/cholesterol/bovine brain phosphatidylserine | Extrusion |
| Farmer and Gaber 1987 | DMPC/cholesterol/dicetylphosphate | |
| Kato et al. 1984 | EYL/carboxymethyl chitin. | Reverse phase evaporation |
| Hunt et al. 1985 | EYPC/cholesterol/DPPA/α-tocopherol | Reverse phase evaporation and Extrusion |
| Hayward et al. 1985 | Diacetylene phospholipid/cholesterol UV-irradiation for polymerization | HbCO, sonication |
| Beissinger et al. 1986 | HSPC/cholesterol/dicetylphosphate or DMPG | Microfluidizer |
| Rudolph et al. 1988 | HSPC/cholesterol/DMPG/α-tocopherol. Trehalose is added | Bovine Hb |
| Rabinovici et al. 1993 | to store LEH as a lyophilized powder | Thin film hydration and emulsification |
| Jopski et al. 1989 | EYL/PS (EYPA) | Detergent dialysis |
| Yoshioka 1991 | HSPC/cholesterol/myristic acid/α-tocopherol/DPPE-PEG | Microfluidizer |
| Takahashi 1995 | | |
| Mobed and Chang 1991 | HSPC/DMPG/α-tocopherol/carboxymethyl chitin | Reverse phase evaporation |
| Sato et al. 1992 | DODPC/cholesterol/octadecadienoic acid | HbCO, Extrusion method |
| Sakai et al., 1992 | Gamma-ray polymerization | |
| Akama et al. 2000 | | |
| Liu and Yonetani 1994 | EYL/cholesterol/dicetylphosphate/α-tocopherol | Freeze–thaw method |
| Sakai et al. 1996 | DPPC/cholesterol/DPPG or palmitic acid | HbCO, extrusion |
| Takeoka et al. 1996 | | |
| Sakai et al. 1997 | DPPC/cholesterol/DPPG/DSPE-$PEG_{5000}$ | HbCO, extrusion |
| Phillips et al., 1999 | DSPC/cholesterl/$PEG_{5000}$-DSPE/a-tocopherol | α-crosslinked human Hb microfluidizer |

(continued)

**Table 12.2** (continued)

| Authors | Lipid composition | Characteristic preparation methods |
|---|---|---|
| Sou et al., 2003<br>Sakai et al. 2002 | DPPC/cholesterol/DHSG/DSPE-$PEG_{5000}$ | HbCO, extrusion |
| Li et al. 2005 | DMPC/cholesterol/DMPG/DSPE-$PEG_{2000}$/actin | Extrusion |
| Pape et al. 2008 | HSPC/cholesterol/stearic acid/DSPE-$PEG_{5000}$ | Lipid paste rapid dispersion |
| Centis and Vermette 2008 | DSPC/cholesterol/palmitic acid/DSPE-$PEG_{2000}$ | HbCO, thin film hydration and extrusion |
| Agashe et al. 2010 | DSPC/cholesterol/CHHDA/DSPE-$PEG_{5000}$/α-tocopherol | HbCO<br>emulsification |
| Rameez et al. 2012 | DSPC/cholesterol/DSPE-$PEG_{5000}$ | Bovine HbCO<br>Thin film hydration and emulsification |

Abbreviations in this table
*DMPC* 1, 2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine
*EYPC* Egg yolk phosphatidylcholine
*DPPA* 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid
*HSPC* Hydrogenated soy phosphatidylcholine
*DMPG* 1, 2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol
*EYL* Egg yolk lecithin
*PS* Phosphatidylserine
*DODPC* 1, 2-dioctadecadienoyl-*sn*-glycero-3-phosphatidylcholine
*DPPE* 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine
*DSPE* 1, 2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine
*DPPC* 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine
*DHSG* 1, 5-*O*-dihexadecyl-*N*-succinyl-L-glutamate
*HbCO* Carbonylhemoglobin
*CHHDA* 2-Carboxyheptadecanoyl heptadecylamide

developed freeze-dried powder LEH from the 1980s (Gaber et al. 1983), but the laboratory terminated its development in the late 1990s (Flower and Rudolph 1999), presumably because of low Hb encapsulation efficiency and induction of anaphylactoid reactions (Szebeni et al. 1999), despite the important LEH advantage of long-term storage as a freeze-dried powder using cryoprotectant saccharides. Terumo Corp. (Japan) started development of Neo Red Cells from around 1985 (Suzuki et al. 1988; Takahashi 1995; Pape et al. 2008) using particles that had been surface-modified with PEG chains. However, it suspended its preclinical studies in 2012. As Table 12.2 shows, most research groups use lipid composition of phosphatidylcholine, cholesterol, negatively charged lipid, and PEG-lipid. Cholesterol not only improves membrane stability; it also reduces the curvature for large unilamellar vesicles. Addition of a small amount of negatively charged lipid increases the repulsive force between the lipid membranes and reduces the lamellarity in addition to controlling the zeta potential for blood compatibility. Saturated phospholipids, such as HSPC, DSPC, and DPPC in Table 12.2, are preferred to unsaturated lipids such as EYL and soy phosphatidylcholines because of the synergistic, facilitated oxidation of both unsaturated lipids and Hb and physical instability (Szebeni et al. 1985), but cholesterol lowers such Hb denaturation to some degree. Utilization of carbonylhemoglobin (HbCO) is effective to prevent denaturation of Hb during preparation procedures.

Our academic consortium has worked to improve the encapsulation efficiency and particle size distribution from the viewpoint of molecular assembly by regulating the electrostatic and hydrophobic interactions between the components (Hb and lipids) (Sakai et al. 2009a). The resulting Hb-vesicles (HbV) encapsulate nearly 30,000 Hb molecules (35 g/dL Hb solution) within a 5 nm thin lipid membrane. The selection of lipids was also important for stability and biocompatibility. The starting material, Hb solution, is purified from outdated NAT-inspected red blood cells provided by the Japanese Red Cross. Bovine Hb and swine Hb are also available for the preparation of HbV (Sakai et al. 2002). Carbonylation of Hb (HbCO) prevents metHb formation and denaturation of Hb, and enables pasteurization at 60 °C for 10 h, thereby ensuring the utmost safety from infection. HbCO encapsulated in HbV can be converted easily to $HbO_2$ by photodissociation using illumination of visible light under $O_2$ atmosphere. We formerly used polymerizable phospholipids (containing dienoyl group in acyl chain) to stabilize the resulting encapsulated Hb because it was believed that liposome had a fragile structure. However, the problem was that the polymerized liposome was so stable that it was not degraded and it remained in the liver and spleen after intravenous administration into rats. Now we use other combination of conventional phospholipid (DPPC), cholesterol, negatively charged synthetic lipid (Sou and Tsuchida 2008), and PEG-conjugated phospholipid. The resulting liposome sufficiently prevents aggregation. Complete deoxygenation of the HbV suspension enables long-term storage for years at room temperature (Sakai et al. 2000). Without decarbonylation, HbCO is stable. It can be stored for a long time. Moreover, injection of a cellular HBOC as an HbCO form is beneficial for some pathological conditions (Sakai et al. 2009) and should be studied intensively.

Details of in vivo results of safety and efficacy of HbV are summarized in some review papers (Sakai et al. 2008; Tsuchida et al. 2009; Sakai et al. 2011). The in vivo oxygen transport capacity of HbV as a resuscitative fluid is described by Dr. Horinouchi in this book.

## 12.4 Advantages of Gas Reactions of Encapsulated Hbs

One important physiological aspect of cellular type HBOCs is that their particles are much larger than those of cell-free HBOCs. They do not seem to induce vasoconstriction or hypertension (Nakai et al. 1998; Sakai et al. 2000). Physiochemical analysis of NO reactions of a series of cell-free HBOCs solutions showed that NO binding rate constants are fast and mostly identical to that of stroma-free Hb (Rohlfs et al. 1988). However, one cellular type of HBOCs, Hb-vesicles (HbV), showed retarded NO binding because of the formation of intracellular diffusion barrier of NO simply by encapsulation of a concentrated Hb solution (Sakai et al. 2008b, 2009b). In fact, HbV encapsulating a diluted Hb solution provides a larger NO binding rate constant: a value similar to that of stroma-free Hb solution.

Moreover, a larger particle shows a slower lateral diffusion in an arteriole that retards the gas reaction at a vascular wall (Sakai et al. 2010). HbV showed a lower rate of NO binding, CO binding, and $O_2$ release in the model vessels, each of which relates to the vascular tone. In addition, the larger particles prevent penetration across the perforated endothelium to approach to a space between the endothelium and the smooth muscle where NO is produced to bind to soluble guanylate cyclase. In fact, RBCs showed the slowest rate of NO binding, CO binding, and $O_2$ release. These data imply that RBCs are evolutionally designed to retard gas reactions in blood circulation.

## 12.5 Intrinsic Difficulties to be Considered for Realization of Encapsulated Hb

Even though Hb encapsulation might shield all the toxic effects of cell-free Hb, cellular HBOCs have their own hurdles that impede their realization. Several are explained here.

### *12.5.1 Particle Size and Encapsulation Efficiency*

The RBC structure is deformable, facilitating its flow through a capillary with a narrower diameter. However, that attribute of deformability is difficult to mimic artificially. Accordingly, the particle should be smaller than the capillary diameter.

It is important to encapsulate a concentrated Hb solution in the particle. To improve the particle function, the weight ratio of the encapsulated Hb to the capsular material is one parameter that must be considered. The Hb concentration in blood is around 12–15 g/dL. A fluid of a cellular HBOC dispersion should have a comparable Hb concentration if it is intended for use as a blood substitute. For this purpose, the intracellular Hb concentration must be as high as intracellular Hb concentration of RBCs, which is around 35 g/dL.

### *12.5.2 Stability of the Capsule*

The capsule should be stable to retain Hb inside the capsules during storage for a long time, and after injection in the blood circulation until it disappears, because elimination of cell-free Hb is the purpose of Hb encapsulation. The encapsulated Hbs are usually captured by the reticuloendothelial system (RES). The capsule material should be degradable in the macrophage. Their components and their degraded or metabolic materials should never be deposited for a long time in the organs. Accordingly, the capsule material should have both stable and unstable characteristics. The pharmacokinetics of both Hb and capsule should be examined (Taguchi et al. 2009).

Trace amounts of ascorbic acid and thiol compounds are present in plasma, and oxidized cell-free HBOCs can be reduced by these compounds. Because of the stability of a capsule, ionic transport through the capsular membrane is shielded to some degree in the absence of a substitute for ion channels. Encapsulated Hb autoxidizes to form metHb and loses its oxygen binding ability. A remedy for such metHb formation must be considered, such as establishing a reduction system in the capsules (Chang T et al. 2000; Tsuchida et al. 2009).

### *12.5.3 Blood Compatibility of the Capsule*

Some of the liposomal products for anticancer therapy induce complement activation. The so-called injection reaction is being clarified continually as clinical experience accumulates, such as dyspensa, tachypenia, tachycardia, hypotension and hypertension, chest pain, and back pain (Szebeni 2005). We confirmed that our prototype HbV, containing phosphatidyl glycerol, induced marked anaphylactoid reactions and cardiopulmonary disorders, manifested as systemic and pulmonary hypertension, increased vascular resistance, decreased cardiac output, thrombocytopenia, tachycardia, etc. (Sakai et al. 2012). Therefore, it is extremely important to confirm the absence of complement activation of the capsule material (Chang and Lister 1994).

Because the cellular type HBOCs are not dissolved but dispersed in the fluid, the particles sometimes aggregate in the presence of plasma protein by ionic

interaction, or depletion interaction. Accordingly, the particle surface would need some surface modification to prevent aggregation.

### *12.5.4 Influence on Clinical Instruments*

Light scattering of the particle dispersion, and a stable capsule that cannot be easily destroyed by a detergent, are the chief causes of interference in clinical laboratory tests based on colorimetric and turbidimetric analysis (including quantitative measurement of Hb in blood) and in clinical diagnostic tools such as laser pulsed oxymetry. The level of interference effect should be examined carefully, and a remedy should be considered in advance (Sakai et al. 2003; Suzaki et al. 2008).

Another important point to be considered includes impacts of the RES trap after a massive dose of cellular HBOCs, which might include transient and local immunosuppression (Takahashi et al. 2011). This point was discussed at length by our collaborators in other chapter (Azuma et al.) in this book. Even though cellular HBOCs are more complicated than cell-free HBOCs, resolving the issues presented above can realize the successful development of cellular HBOC.

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**Disclosure** Hiromi Sakai is an inventor holding some patents related to the production and utilization of Hb-vesicles.

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# Chapter 13
# Recombinant Octameric Hemoglobins as Resuscitation Fluids in a Murine Model of Traumatic Brain Injury Plus Hemorrhagic Shock

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## Abbreviations

| | |
|---|---|
| HBOC | Hemoglobin-based oxygen-carrier |
| Hb | Hemoglobin |
| rHb | Recombinant hemoglobin |
| TBI | Traumatic brain injury |
| CCI | Controlled cortical impact |
| HS | Hemorrhagic shock |
| ICP | Intracranial pressure |
| $PbtO_2$ | Brain tissue oxygen concentration |
| MAP | Mean arterial blood pressure |
| LR | Lactated Ranger's solution |
| DSS | 2, 2-dimethyl-2-silapentene-5-sulfonate |
| rHb (αN78C) | Recombinant hemoglobin with Asn78 to Cys substitution on the α-subunit |
| rHb (αN78C/L29F) | Recombinant hemoglobin with Asn78 to Cys and Leu29 to Phe substitutions on the α-subunit |

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| | |
|---|---|
| rHb (αN78C/L29W) | Recombinant hemoglobin with Asn78 to Cys and Leu29 to Trp substitutions on the α-subunit |
| Iso | Isofluorane |

## 13.1 Introduction

Tremendous effort has gone into developing formulations to substitute red blood cells transfusion. Most of these formulations are hemoglobin-based oxygen-carriers (HBOCs) [See (Natanson et al. 2008) for review]. Acellular hemoglobin (Hb) is cytotoxic by reacting with endothelium-derived NO to form bioinactive nitrate (Minneci et al. 2005), which may lead to vascular thrombosis of the heart and other organs (Rother et al. 2005). Furthermore, Hb without the confine of red blood cells is prone to dissociation into dimers, with subsequent loss in oxygen-binding activity and allostery (Baudin-Creuza et al. 2011). Hence, hemoglobins have been cross-linked (Chatterjee et al. 1986), polymerized (Sehgal et al. 1984) or surface-conjugated (Conover et al. 1996; McCarthy et al. 2001) to create larger and more stable molecules based on the postulation that larger HBOCs could prevent extravasation and have lower toxicities. With the advent of protein engineering, recombinant hemoglobin (Shen et al. 1993, 1997) can be prepared in sufficient quantity for HBOC studies. By substituting protein surface residue(s) with cysteine(s), polymeric (Fronticelli et al. 2001) or octameric (Fablet et al. 2003; Vasseur-Godbillon et al. 2006) hemoglobins can be generated. Furthermore, Hb molecules with unique oxygen-binding and/or autoxidative properties can be obtained by mutating certain amino acid residue(s) on the protein. For instance, replacing the Leu29 residue of the α-subunit (αL29) with phenylalanine inhibits oxidation accompanied by an increase in oxygen affinity (Eich et al. 1996; Olson et al. 1997; Jeong et al. 1999). However, substituting the αL29 residue with tryptophan produces a tetramer with low oxygen affinity (Wiltrout et al. 2005). Nonetheless, octameric hemoglobins possessing these interesting properties have not been tested *in vivo* with any clinically relevant models.

Traumatic brain injury (TBI) is an important public health problem world-wide. Outcomes from TBI are substantially worsened by secondary insults, such as hemorrhagic shock (HS), which occurs in approximately 30 % of TBI victims (Chesnut et al. 1993). In experimental models, TBI combined with HS can cause neuronal death in the hippocampus beneath the cerebral contusion at mild injury levels where no neuronal death is seen in TBI alone. The situation poses a special challenge to the field of resuscitation medicine. A number of strategies have been explored with limited success (Bhardwaj and Ulatowski 2004; Brasel et al. 2008; Doyle et al. 2001; Forsyth et al. 2008). Blood substitutes, however, may present a promising strategy for resuscitation of patients with TBI plus HS. Recent studies suggest resuscitation with solutions of polymerized hemoglobin provided substantial benefits on intracranial pressure (ICP), brain tissue oxygenation ($PbtO_2$),

and neuropathology (Patel et al. 2006; Rosenthal et al. 2008; Stern et al. 2009). Unfortunately, enthusiasm for blood substitutes was blunted considerably when multiple clinical trials concluded that current generation of HBOCs increased incidence of myocardial infarction and mortality (Natanson et al. 2008). Hence, a better Hb-based resuscitation solution is desperately needed for polytrauma casualties suspected of suffering TBI.

Given the failures of HBOCs in clinical trials (Natanson et al. 2008), it seems appropriate to consider a paradigm shift in the approach to the development of blood substitutes. We believe that this paradigm shift should incorporate two strategies. First, it should develop novel and targeted HBOCs that are molecularly and/or chemically designed not only to minimize toxicities, but also such that HBOC performance is optimized for specific indications. For example, in the setting of shock, benefit might be maximized using an HBOC that not only features reduced NO binding but also exhibits specific oxygen affinity characteristics and/or other modifications critical to the disease process being targeted. Recombinant Hbs represent perfect candidates in this regard. Using this methodology, one has the opportunity to design a Hb molecule with designed oxygen affinity, reduced rate of autoxidation, NO binding affinity, etc. Olson and colleagues have written an excellent review on the design of recombinant Hbs for HBOCs with emphasis on autoxidation and NO binding affinity (Varnado et al. 2012). Second, we posit that pre-clinical studies should be carried out in experimental models that more closely mimic the complex clinical scenarios that have the greatest need for HBOC therapies. For example, when polytrauma and HS include TBI, hypotensive resuscitation, which prevents "blood washout" in uncontrolled hemorrhage shock, may be inadequate for the injured brain—which has high metabolic demands early after the injury (Bauman et al. 2009). TBI guidelines (Bratton et al. 2007) recommend avoiding hypotension in this setting which is quite different from HS alone. And in TBI plus HS, resuscitation with large volumes of crystalloid can exacerbate brain edema and intracranial hypertension. Thus, TBI plus HS represents a specific form of polytrauma that could greatly benefit from an HBOC. TBI plus HS may also represent a special opportunity for HBOC therapy because it generally occurs in the young, and is of importance in combat casualty care from blast TBI in service persons who are, once again, young and free of cardiovascular diseases. These trauma victims may develop the fewest side effects from HBOC therapy and thus may be best for initial demonstration of efficacy (Elmer et al. 2012).

In this communication, we report on the administration of three octameric hemoglobins as resuscitation solutions in mice after experimental TBI plus HS. The systemic hemodynamics, resuscitation volume requirements, survival, $PbtO_2$, and neuropathology of the mice were assessed after treatment. The recombinant octameric hemoglobin derives from substituting a surface asparagine at the 78 position of the α-subunit with cysteine (αN78C). The rHb (αN78C) remains as octamers in the presence of human plasma (Brillet et al. 2012). Its large molecular size offers the possibility of diminished NO binding. In addition, the leucine residue locates in the distal heme pocket (B10 helix) of the α-subunit was mutated to either phenylalanine or tryptophan to generate mutants with high- [rHb (αN78C/

L29F)] and low-oxygen [rHb (αN78C/L29W)] affinities, respectively, when compared to that of Hb A. Our results demonstrate that these octameric rHbs can serve as small volume resuscitation solutions in mice suffering TBI with HS, compared to conventional lactated Ringer's (LR) solution. Acute hemodynamic effects of these rHbs may be predicted based on theoretical effects on NO binding. Increasing Hb oxygen affinity also produces a trend towards lower $PbtO_2$ and surprisingly, reduced neuronal death in the CA1 region of the hippocampus, a brain region that is highly sensitive to ischemic insults.

## 13.2 Materials and Methods

### *13.2.1 Materials*

Hb A was isolated from human normal blood samples obtained from local blood bank with a published protocol from our laboratory (Russu et al. 1984). Restriction enzymes were purchased from New England BioLabs. QuikChange site-directed mutagenesis kit was a product of Stratagene. All other chemicals are of reagent grade and obtained from Sigma unless specified.

### *13.2.2 Expression and Purification of rHbs*

Construction of the plasmid pHE2073 that encodes rHb (αN78C) for co-expression with methionine aminopeptidase has been reported (Baudin-Creuza et al. 2011). With standard molecular biology techniques, the pHE2073 plasmid was used as template in polymerase chain reactions with appropriate mutation primers to replace the Leu29 residue of the α-subunit (αL29) with either phenylalanine or tryptophan. The resulting pHE2053 and pH2054 plasmids encode rHb (αN78C/L29F) and rHb (αN78C/L29W), respectively.

These plasmids were transformed separately into *E. coli* JM109 for protein expression. Transformed cells were cultured in a 20 L fermentor (B. Braun Biotech International, model Biostat C) in DM-4 medium (Looker et al. 1994) at 32 °C. Glucose in the medium was maintained at 0.8–1 % throughout the culturing period. Recombinant protein expression was induced with 0.1 mM isopropyl *β*-thiogalactopyranoside (IPTG) for 6 h when the culture reached an optical density of 10 at 600 nm. Hemin (25 mg/L) was added to the culture at 0 and 3 h after IPTG induction. At the end of the induction period, cells normally reached an optical density of 30 or higher at 600 nm. Cells were then collected by centrifugation and the wet paste was stored at −80 °C.

The recombinant proteins were purified as published (Shen et al. 1997; Wiltrout et al. 2005) with minor modifications at 4 °C under CO environment. Briefly, cells

were suspended in Buffer A (40 mM Tris–HCl, pH 8.6, and 1 mM benzamidine) at 3 g/mL. Cell lysis was carried out in a high-pressure homogenizer (Avestin, EmulsiFlex-C3). Cell debris and large DNA fragments were removed by centrifugation at 22,000 x g for 2.5 h. Polyethyleneimine was then added to a concentration of 0.5 % and the precipitated nucleic acids were removed by centrifugation at 15,000 x g for 30 min. The sample was concentrated with a Vivaflow 200 system (Sartorius Stedim Biotech GmbH), then dialyzed overnight against Buffer B (20 mM Tris–HCl, pH 8.6, 0.5 mM EDTA). The recombinant proteins were loaded onto a Q-Sepharose Fast-Flow (GE Healthcare Life Sciences) column equilibrated and washed with Buffer B. Proteins were eluted from the column with Buffer C (20 mM Tris–HCl, pH 6.5, 0.5 mM EDTA) then oxidized for 1 h with four-fold molar excess of $K_3Fe(CN)_6$. Excess chemicals were removed by gel permeation through a Sephadex G-25 column with Buffer D (50 mM sodium phosphate, pH 6.8) as carrier. The sample was incubated overnight at room temperature then reduced with four-fold molar excess of sodium dithionite dissolved in Buffer D under nitrogen. The reduced protein sample was loaded immediately onto a Sephadex G-25 column that has been equilibrated with Buffer E (10 mM sodium phosphate, pH 6.8, 0.5 mM EDTA) freshly equilibrated with CO and eluted with the same buffer. Final purification was carried out on a Mono S 16/10 column (GE Healthcare Life Sciences) equilibrated in Buffer E and eluted with Buffer F (20 mM sodium phosphate, pH 8.3, 0.5 mM EDTA) at an increment of 2 % Buffer F per column volume. The octamers eluted off the column at approximately 22–25 % Buffer F. The molecular weights of the rHb subunits were affirmed with mass spectrometry and the amount of N-terminal methionine cleavage was estimated with Edman degradation (Shen et al. 1993, 1997). All rHbs used in this study had the correct molecular weights and less than 5 % N-terminal methionine.

For rHbs used as resuscitation solutions, they were converted to oxy-Hb then passed through an EndoBind-R column (BioDtech, Inc) for endotoxin removal. Samples were collected in pyrogen free test tubes (Lonza Walkersville, Inc.) and further concentrated to 120 mg/mL with ultrafiltration columns (Vivaspin, Satorius Stedim Biotech GmbH) for studies.

### *13.2.3 Oxygen-Binding Measurements*

Data were acquired on a Hemox Analyzer (TCS Medical Products) at 29 °C as a function of pH in 0.1 M sodium phosphate (Shen et al. 1993, 1997). The solution contained 0.1 mM hemoglobin (in terms of heme) and a methemoglobin (met-Hb) reductase system to slow down the formation of met-Hb (Hayashi et al. 1973). Results from each equilibrium binding curve were fit to the Adair equation and oxygen affinity was determined from the $P_{50}$ value (in millimeters of Hg) which was taken at 50 % $O_2$ saturation. The cooperativity of the hemoglobin samples was

estimated with the Hill coefficient ($n_{50}$), which was calculated from the slope of the Hill plot at 50 % saturation and had an accuracy of ±10 %.

### 13.2.4 Structural Study with $^1$H NMR Spectroscopy

$^1$H NMR spectra were collected on Bruker Avance DRX-300 or DRX-600 spectrometer. Samples were 5 % (3.1 mM in terms of heme) hemoglobin in 0.1 M sodium phosphate buffer at pH 7.0 containing 95 % $H_2O$ and 5 % deuterium oxide ($D_2O$). Experiments were performed at 29 °C. A jump-and-return pulse sequence was used to suppress the water signal (Plateau and Gueron 1982). Proton chemical shifts are referenced to the methyl proton resonance of 2, 2-dimethyl-2-silapentene-5-sulfonate (DSS) indirectly by using the water signal at 4.76 ppm downfield of DSS at 29 °C.

### 13.2.5 Mouse Model of Traumatic Brain Injury Combined with Hemorrhagic Shock

C57BL6 mice (Jackson Laboratories) 12–15 weeks of age and weighing of 27.7 ± 0.5 g were housed in controlled environmental condition. Food and water were availed ad *libitum* until the start of the study. Induction of TBI by controlled cortical impact (CCI) with subsequent hemorrhage was carried out according to (Hemerka et al. 2012), with modifications. Mice were anesthetized with 4 % isofluorane in 2:1 $N_2O/O_2$ via nose cone. Femoral venous and arterial catheters were inserted via an inguinal cut-down and the animals were placed in a stereotactic frame. A 5 mm craniotomy was then performed and the bone flap removed. The brain temperature was controlled with a heat lamp at 37.0–37.5 °C and monitored with a probe (Physitemp) inserted into the right parietal cortex via a burr hole. The body temperature of the animal was checked with a rectal probe.

CCI was performed with a pneumatic impactor (Bimba) using a flat 3 mm tip at a velocity of 5 m/s to a depth of 1 mm. TBI at this level without HS produced a mild to moderate level of injury with no appreciable loss of CA1 hippocampal neurons and no mortality (Dennis et al. 2009). A tissue $PO_2$ probe was then inserted via the CCI craniotomy with its tip stayed beneath the CCI injury and approximate to the core of the hippocampus. The initial reading 5 min after insertion was taken as “baseline” and subsequent readings were converted to percent of baseline. Anesthesia was then changed to 1.5 % isofluorane in room air for 10 min, after which baseline hemodynamic and $PbtO_2$ readings were recorded.

Blood was withdrawn from the mice via the femoral arterial catheter over 15 min to a total volume of 2.4 mL/100 g body weight. Over the next 20 min, the mean arterial blood pressure (MAP) of the mice was maintained between 25–27 mm Hg to mimic HS, via further withdrawal or reinfusion of blood as needed. The HS phase was 35 min in length.

### 13.2.6 *Experimental Protocol for* In Vivo *Testing*

The experimental protocol is detailed in Fig. 13.1. The time course includes a 35 min HS Phase, a 90 min "Pre-Hospital" resuscitation Phase, a 15 min "Hospital" resuscitation Phase, and a 24 h Observation Phase. The Pre-Hospital Phase was initiated by administering a bolus of LR or rHbs (12 % solution in terms of heme) at 2 mL/100 g body weight. LR was then infused continuously at 2 mL/100 g/hr as a maintenance fluid. Additional LR was given at 1 mL/100 g/5 min if MAP of the animal decreased below 70 mm Hg. After 90 min of "Pre-Hospital" resuscitation, the Hospital Phase was started with 100 % oxygen and return of all shed blood to mimic the definitive care in an emergency room. At the end of this phase, all catheters and probes were removed and the animals were sutured. Mice were returned to cage and observed for 24 h, then sacrificed for neuropathology by perfusion fixation. Blood pressure and $PbtO_2$ were assessed continuously in the

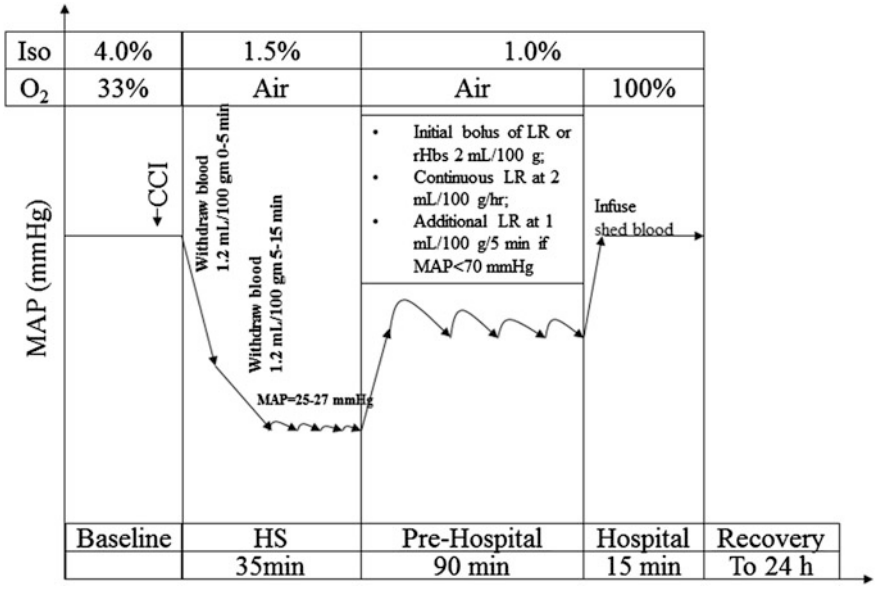


**Fig. 13.1** Experimental protocol for studies of combined traumatic brain injury (TBI) and hemorrhagic shock (HS) designed to mimic the clinical scenario of a polytrauma victim, with TBI and a secondary insult from severe hemorrhagic hypotension. TBI, was induced using the controlled cortical impact (CCI) model in C57BL6 mice at a mild-moderate injury level. This was followed by severe pressure controlled HS (mean arterial blood pressure [MAP] at 25–27 mm Hg) induced by blood withdrawal for a period of 35 min. To mimic pre-hospital resuscitation, a 90-min period followed with administration of either lactated Ringers (LR) solution or recombinant hemoglobin (rHb). A 15-min phase mimicking hospital emergency department care followed during which time the shed blood was re-infused and pure oxygen was administered. Anesthesia in the model was provided using isoflurane (Iso). This model thus presents a highly clinically relevant scenario for testing novel resuscitation solutions such as rHbs

hippocampus ipsilateral to the CCI. Arterial blood gases were determined after CCI (baseline), and at the end of each phase. Total blood Hb levels were determined using Hemocue 201 (Hemocue Inc.) which measured Hb in both erythrocytes and plasma. The number of animals used in our study with resuscitation fluids was: LR (n = 15); rHb (αN78C) (n = 7); rHb (αN78C/αL29F) (n = 9); and rHb (αN78C/αL29W) (n = 7). Differences in sample size related to differences in availability of the various rHbs.

### *13.2.7 Histology*

Mice were anesthetized with 4 % isofluorane and by transcardial perfusion with ice-cold saline followed by 10 % buffered formalin. Brain tissues were fixed with 10 % buffered formalin and embedded in paraffin. Multiple 5 mm sections, 200 mm apart from bregma −1.86 to −2.26, were prepared from each brain. Sections were stained with hematoxylin and eosin (Thermo Scientific). The number of surviving neurons in the CA1 and CA3 regions of the hippocampus were quantified by a blinded observer in the hematoxylin and eosin stained sections (ImageJ software, NIH). Neuron counts were quantified as densities per 100 μm segments of the hippocampal subfields.

### *13.2.8 Statistical Analysis*

Data are presented as mean ± standard error unless it was stated otherwise. Analysis of variance was used to compare continuous physiologic variables between groups. Appropriate post hoc analysis was performed using the Student–Newman–Keuls (for all group-wise comparisons) or Dunnetts test (for comparison to LR resuscitation control). Kruskal–Wallis tests were used to determine the significance of non-parametric data.

## 13.3 Results

### *13.3.1 rHb Characterization*

#### 13.3.1.1 Oxygen-Binding Properties

The oxygen-binding properties of the rHb mutants were determined and compared to that of Hb A in 0.1 M phosphate buffer as a function of pH at 29 °C. The properties of mouse Hb were also determined and plotted together for direct

comparison. The rHb (αN78C) mutant has similar $P_{50}$ values as that of Hb A between pH 5.7 to pH 8.5 (Fig. 13.2a). The results agree closely to that of Brillet et al. (2012) and Baudin-Creuza et al. (2011) and show clearly that the substitution of a surface residue, αAsn78, with Cys does not change the function of the macromolecule. However, additional replacement of the B10 residue (αLeu29) alters the oxygen-binding affinity of the protein. The rHb (αN78C/L29F) and the rHb (αN78C/L29W) mutants have low and high $P_{50}$ values, respectively. Data indicate rHb (αN78C/L29F) is a mutant with high oxygen affinity while rHb(αN78C/L29W) is a low oxygen affinity mutant. The mouse Hb has $P_{50}$ values higher than those of Hb A, but substantially lower than those of the rHb (αN78C/L29W) mutant (Fig. 13.2a).

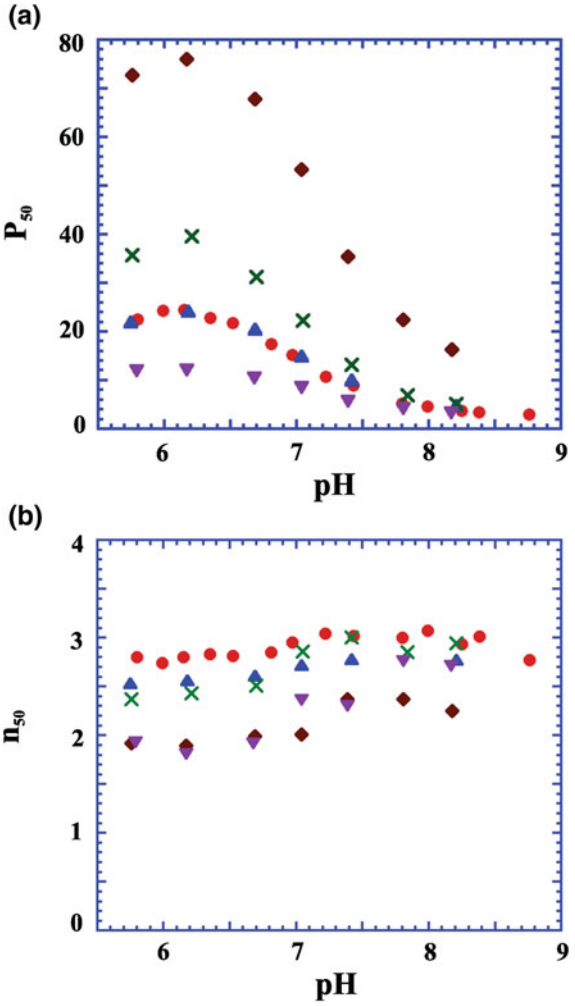


**Fig. 13.2** Oxygen binding properties of Hb A, octameric rHbs, and mouse Hb in 0.1 M sodium phosphate buffer as a function of pH at 29 °C: **a** Oxygen binding as a function of pH and **b** Hill coefficient as a function of pH. (*dot*) Hb A, (*X*) mouse Hb, (*triangle*) rHb (αN78C), (*inverted triangle*) rHb (αN78C/L29F), and (*diamond*) rHb (αN78C/L29W)

The Hill coefficients ($n_{50}$), a measure of the cooperative oxygenation process, of Hb A, mouse Hb, and the rHbs are plotted in Fig 13.2b. The experimental values obtained for the rHb (αN78C) are lower than those of Hb A, but similar to the results observed for mouse Hb. Mutation in the distal heme pocket further decreases the $n_{50}$ values. Nonetheless, all the mutants show cooperativity in oxygen binding, even for those containing distal heme pocket substitution.

#### 13.3.1.2 NMR Spectra of Hbs in the CO Form

$^1$H NMR spectroscopy is an excellent tool to probe tertiary and quaternary structural changes in hemoglobin (Ho 1992). The exchangeable protons in the inter-subunit interfaces give resonance signals between 9 and 14 ppm. The resonances at 12.9 and 12.1 ppm from DSS have been assigned to the $NH_1$ of αHis122 and αHis103, respectively (Russu et al. 1987; Simplaceanu et al. 2000). These two residues locate in the $\alpha_1\beta_1$ interface. The resonance at 10.6 ppm has been assigned to the $NH_{\varepsilon 1}$ of βTrp37 (Simplaceanu et al. 2000; Fang et al. 2000), which is located in the $\alpha_1\beta_2$ interface. Figure 13.3a shows clearly that no noticeable shift has been detected in these resonances among the spectra generated for Hb A and the three mutants. Hence, the quaternary structure at the $\alpha_1\beta_1$ and the $\alpha_1\beta_2$ interfaces has been preserved.

Spectra representing the non-exchangeable ring-current shifted proton resonances from 0 to −3 ppm from DSS are presented in Fig. 13.3b. The resonances at −1.75 and −1.82 ppm from DSS have been assigned to the $\gamma_2$-$CH_3$ group of αVal62 and βVal67, respectively (Lindstrom et al. 1972; Dalvit and Ho 1985). These two residues located on the E helix and the E11Val methyl resonances provide information about the tertiary structure of the heme pockets. As expected, the NMR spectrum representing the rHb (αN78C) mutant is similar to that of Hb A and the resonance at −1.82 ppm has not been shifted for all three mutants. However, the resonance at −1.75 ppm shifted noticeable in the rHb (αN78C/L29F) and the rHb (αN78C/L29W) mutants, but not the rHB (αN78C) mutant. These results demonstrate clearly the αAsn78 mutation does not change the structure of the heme pockets while the αLeu29 substitutions affect only the tertiary structure of the α-subunit heme pocket.

#### 13.3.1.3 NMR Spectra of Hbs in the Deoxy Form

The hyperfine-shifted proton resonances spectra representing Hb A and the three mutants covering the region 55–80 ppm from DSS are shown in Fig. 13.4a. For deoxy-Hb, the $N_\delta H$ exchangeable proton of αHis87 and βHis92 yield signals at 63 and 76 ppm, respectively (Takahashi et al. 1980). These two histidine residues locate at the proximal heme pockets and any shift in resonance signal indicates a change in the tertiary structure at the heme pocket. There is no shift in these two resonances in rHb (αN78C) compared to Hb A. However, we have detected a

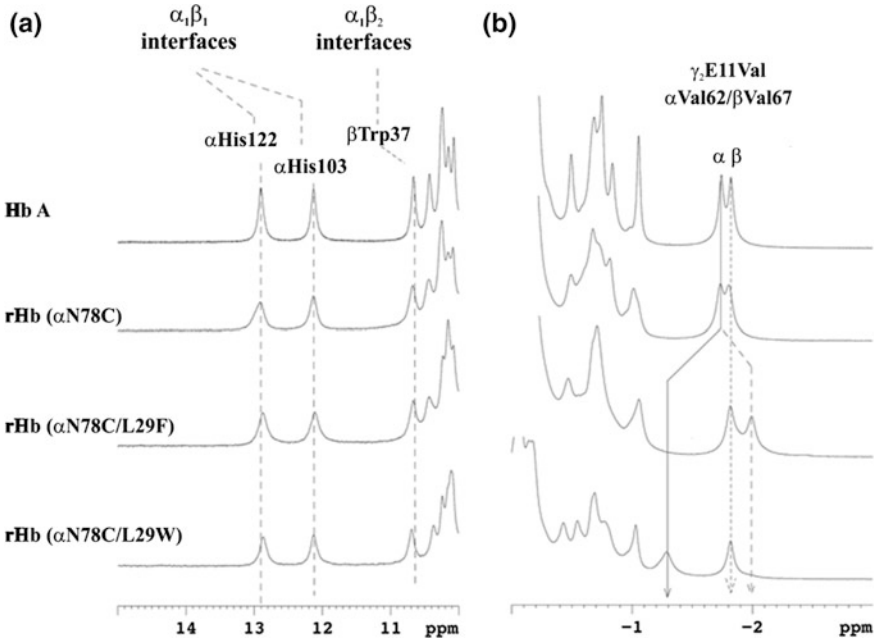


**Fig. 13.3** $^{1}$H NMR spectra (600 MHz) of the CO form of Hb A and octameric rHbs in 95 % H2O, 5 % $D_2O$, 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C: *a* Exchangeable proton resonances and *b* ring current shifted proton resonances

downfield shift of 4–5 ppm for the resonance at 63 ppm while the resonance at 76 ppm remains unaffected for the rHb (αN78C/L29F) and the rHb (αN78C/L29W) mutants. Therefore, the tertiary structure of the proximal heme pocket has been disturbed in the α-subunits of the Hb molecule carrying αLeu29 mutations as expected. The tertiary structure of their *β*-subunit heme pocket remains intact.

The spectra for exchangeable protons in the inter-subunit interfaces are presented in Fig. 13.4b. The resonances at 13.0 and 12.2 ppm from DSS are generated by the $NH_{\varepsilon 1}$ of αHis122 and αHis103, respectively (Ho 1992). We cannot detect any significant shift in these resonances from the mutants. Hence, the quaternary structure of these three octamers in the deoxy form at the $\alpha_1\beta_1$ interface has not been perturbed.

*β*Trp37 is located in the $\alpha_1\beta_2$ interface and forms an H-bond to αAsp94 (Dickerson and Geis 1983). Its exchangeable indole proton gives a resonance signal at 11.2 ppm from DSS. A slight shift of 0.3 ppm was detected for this signal in the rHb (αN78C/L29F) mutant. Another important T-structure marker is the resonance at 14.1 ppm, which is contributed by the H-bond between αTyr42 and *β*Asp99 in the $\alpha_1\beta_2$ interface of deoxy-Hb A (Fung and Ho 1975). We have observed a slight 0.3–0.5 ppm shift for this resonance in both rHb (αN78C/L29F) and rHb (αN78C/L29W) mutants. Combining these results, we conclude that the rHb (αN78C/L29F) and rHb (αN78C/L29W) mutants have slight but noticeable changes in the $\alpha_1\beta_2$ interface.

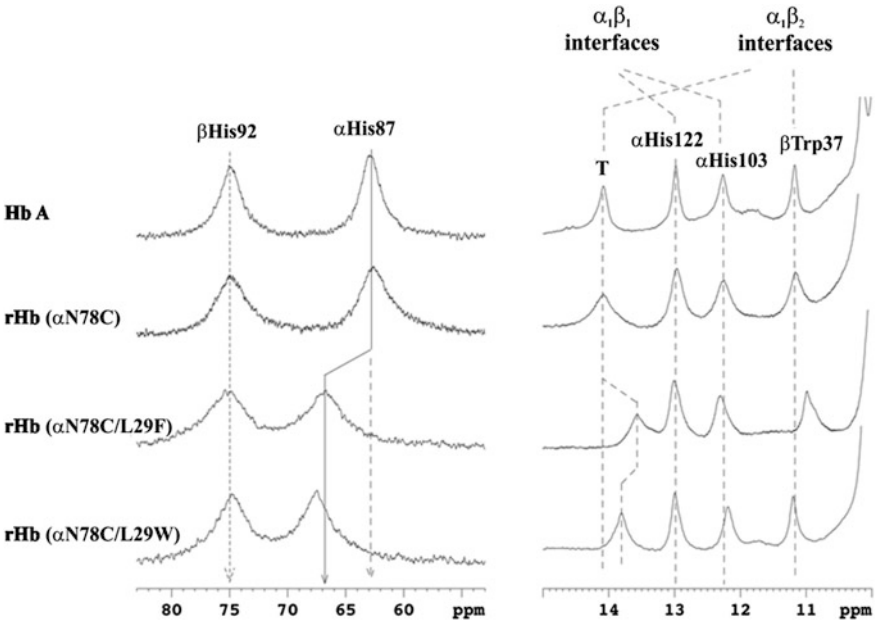


**Fig. 13.4** $^1$H NMR spectra of the deoxy form of Hb A and octameric rHbs in 95 % H2O, 5 % D2O, 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C: **a** Hyperfine-shifted $N_\delta H$ proton resonances of proximal histidyl residues and **b** exchangeable protein resonances

## *13.3.2 rHb as a Resuscitation Solution*

### 13.3.2.1 Endotoxin Level in Octameric rHbs

The purified octameric rHbs were further isolated from an EndoBind-R column for endotoxin removal. Parts of the final products were sent to Lonza Walkersville, Inc for endotoxin analysis. The samples have on the average 131 and 2.43 EU/mL of endotoxin before and after passing through the column, respectively.

### 13.3.2.2 Survival

TBI combined with HS is a severe insult. Nevertheless, mice in all groups can generally survive through the Pre-Hospital Phase (Table 13.1). Statistically, no difference in survival rate was detected among groups considering either survival to the Hospital Phase or to 24 h post CCI ($P = 0.435$, Chi Square test).

### 13.3.2.3 Hemodynamics

MAP was normalized in all rHb groups 5 min after administration of the rHb solution. MAP in these groups stayed near baseline level over the entire Pre-

**Table 13.1** Fluid requirement during pre-hospital phase and survivals

| Groups | Fluid requirements mL/100 g | Survival to hospital phase | Survival to 24 h |
|---|---|---|---|
| LR | 21.5 ± 7.5 | 12/15 | 12/15 |
| rHb (αN78C) (normal $O_2$ affinity) | 5.0 ± 0 | 6/7 | 5/7 |
| rHb (αN78C/L29F) (high $O_2$ affinity) | 5.0 ± 0 | 7/9 | 7/9 |
| rHb (αN78C/L29W) (low $O_2$ affinity) | 5.0 ± 0 | 7/7 | 3/7 |

Hospital Phase (90 min, Fig. 13.5). Furthermore, the rHb groups did not need additional boluses after the initial dosage of 2 mL/100 g body weight of a 12 % rHb solution. In contrast, the LR group had persistent refractory hypotension ($p < 0.01$ vs rHb groups) and additional boluses were needed to maintain the MAP > 70 mm Hg which is consistent with prior reports of this model (Dennis et al. 2009). Consequently, the LR group received 4 times more resuscitation fluid during the Pre-Hospital Phase than that of the rHb groups (Table 13.1). It is of interest to note that an increase in MAP was noted in the LR group during the Hospital Phase after returning all shed blood to the animals. This increment of MAP was not observed in the rHb-treated groups. Among the rHb-treated groups,

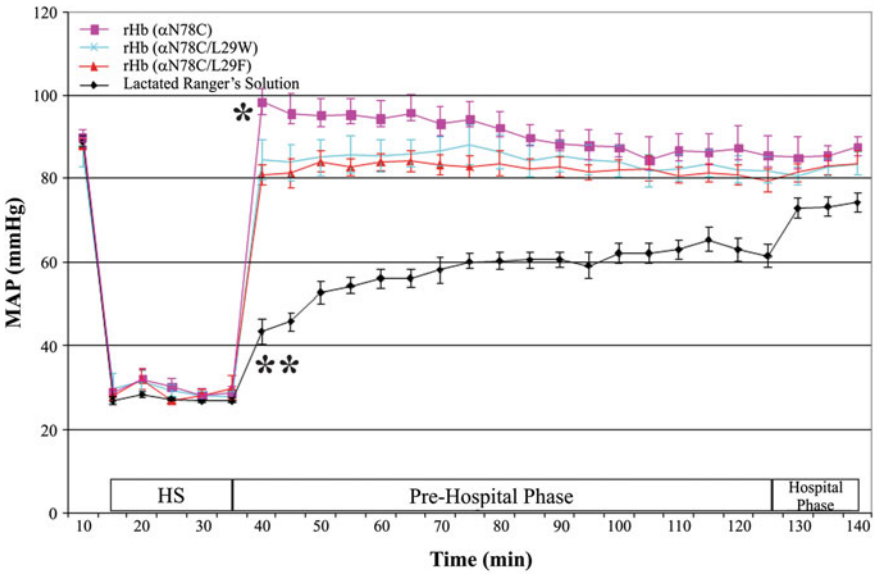


**Fig. 13.5** Mean arterial blood pressure (MAP) during HS, Pre-Hospital and Hospital Phases. *P < 0.05 for rHb αN78C vs rHb αN78C/L29F and rHb αN78C/L29W; **P < 0.01 for LR vs all rHbs. Please see text for details

the initial elevation in MAP was significantly higher in the group receiving rHb (αN78C) (Fig 13.5, $p < 0.05$ vs other 2 rHb groups).

#### 13.3.2.4 Arterial Blood Gas Analysis and Hemoglobin Levels

Arterial blood gas values do not differ among the 4 treatment groups at baseline. At the end of the Pre-Hospital Phase, Hb levels in the rHb groups were approximately 3 g/dL higher than that of the LR group ($p < 0.05$, Table 13.2). This significant increase in Hb for all of the rHb-treated groups versus the LR-treated group also remained at the end of the Hospital phase—i.e., after the shed blood had been re-infused (Table 13.2). All groups had almost normalized arterial blood gas and lactate levels and no difference is detected among groups at any time point

**Table 13.2** Arterial blood gas analysis at each phase of the study

| | Baseline | End of HS | End of Pre-hospital phase | End of hospital phase |
|---|---|---|---|---|
| **pH** | | | | |
| Lactated Rangers | 7.42 ± 0.01 | 7.33 ± 0.01 | 7.40 ± 0.01 | 7.33 ± 0.02 |
| rHb (αN78C) | 7.41 ± 0.01 | 7.36 ± 0.02 | 7.39 ± 0.01 | 7.34 ± 0.02 |
| rHb (αN78C/L29F) | 7.41 ± 0.01 | 7.34 ± 0.02 | 7.39 ± 0.01 | 7.33 ± 0.01 |
| rHb (αN78C/L29W) | 7.42 ± 0.02 | 7.35 ± 0.03 | 7.40 ± 0.00 | 7.34 ± 0.00 |
| **$PaO_2$ (mm Hg)** | | | | |
| Lactated Rangers | 157.5 ± 4.0 | 87.1 ± 3.1 | 82.6 ± 2.5 | 446.3 ± 21.3 |
| rHb (αN78C) | 150.1 ± 14.3 | 78.1 ± 4.0 | 77.7 ± 4.7 | 452.5 ± 8.6 |
| rHb (αN78C/L29F) | 158.0 ± 3.9 | 86.6 ± 3.6 | 76.6 ± 3.2 | 431.8 ± 18.1 |
| rHb (αN78C/L29W) | 142.8 ± 16.2 | 86.6 ± 3.8 | 76.5 ± 1.7 | 453.4 ± 4.4 |
| **$PaCO_2$ (mm Hg)** | | | | |
| Lactated Rangers | 28.5 ± 1.4 | 27.1 ± 1.1 | 28.1 ± 1.2 | 34.8 ± 2.5 |
| rHb (αN78C) | 27.8 ± 0.6 | 27.7 ± 1.5 | 29.2 ± 1.0 | 34.2 ± 2.3 |
| rHb (αN78C/L29F) | 28.8 ± 0.6 | 25.2 ± 1.5 | 29.1 ± 0.9 | 35.2 ± 1.8 |
| rHb (αN78C/L29W) | 28.1 ± 1.0 | 25.0 ± 1.6 | 27.3 ± 0.6 | 34.9 ± 1.9 |
| **Hb (g/dL)** | | | | |
| Lactated Rangers | 14.6 ± 0.1 | 10.4 ± 0.5 | 6.7 ± 0.5 | 10.5 ± 0.5 |
| rHb (αN78C) | 13.8 ± 0.5 | 10.5 ± 0.4 | 9.9 ± 0.2* | 12.3 ± 0.2* |
| rHb (αN78C/L29F) | 14.0 ± 0.4 | 8.7 ± 0.2 | 9.2 ± 0.3* | 11.7 ± 0.2* |
| rHb (αN78C/L29W) | 14.6 ± 0.1 | 9.5 ± 0.4 | 10.0 ± 0.2* | 12.4 ± 0.2* |
| **Lactate (mmol/L)** | | | | |
| Lactated Rangers | 1.7 ± 0.1 | 4.7 ± 0.6 | 1.8 ± 0.2 | 1.1 ± 0.1 |
| rHb (αN78C) | 1.7 ± 0.2 | 3.4 ± 0.4 | 1.3 ± 0.1 | 1.0 ± 0.1 |
| rHb (αN78C/L29F) | 1.4 ± 0.1 | 5.1 ± 0.4 | 0.9 ± 0.1 | 1.0 ± 0.1 |
| rHb (αN78C/L29W) | 1.7 ± 0.2 | 4.5 ± 0.5 | 1.3 ± 0.1 | 1.1 ± 0.1 |

HS, hemorrhagic shock; rHb (αN78C), normal oxygen affinity recombinant hemoglobin; rHb (αN78C/L29F), high oxygen affinity recombinant hemoglobin; rHb (αN78C/L29W), low oxygen affinity recombinant hemoglobin; $*P < 0.05$ vs respective lactated Rangers solution group (ANOVA and Student-Newman-Keul's test)

(including baseline, end of HS phase, end of Pre-Hospital Phase, and end of Hospital Phase) (Table 13.2).

#### 13.3.2.5 Brain Tissue Oxygen Concentration

At the end of the HS Phase, $PbtO_2$ consistently decreased to approximately 20 % of baseline. Resuscitation with LR or rHb solutions can slowly increase $PbtO_2$, but none of the groups has normalized $PbtO_2$ (Fig. 13.6). Numerically, the group receiving the high oxygen affinity rHb (αN78C/L29F) has the lowest $PbtO_2$ throughout the Pre-Hospital Phase ($P > 0.05$). During the Hospital Phase, when 100 % oxygen was administered and all shed blood re-infused, as anticipated $PbtO_2$ increased to values markedly greater than baseline (results not shown) and again no difference was detected among groups.

#### 13.3.2.6 Neuropathology

As previously reported (Dennis et al. 2009), TBI combined with HS causes significant neuronal death in both CA1 and CA3 regions on the hippocampus ipsilateral to the side of CCI in all groups. Surprisingly, neuronal survival is greatest in CA1 hippocampus in the high oxygen affinity rHb (αN78C/L29F) treated group.

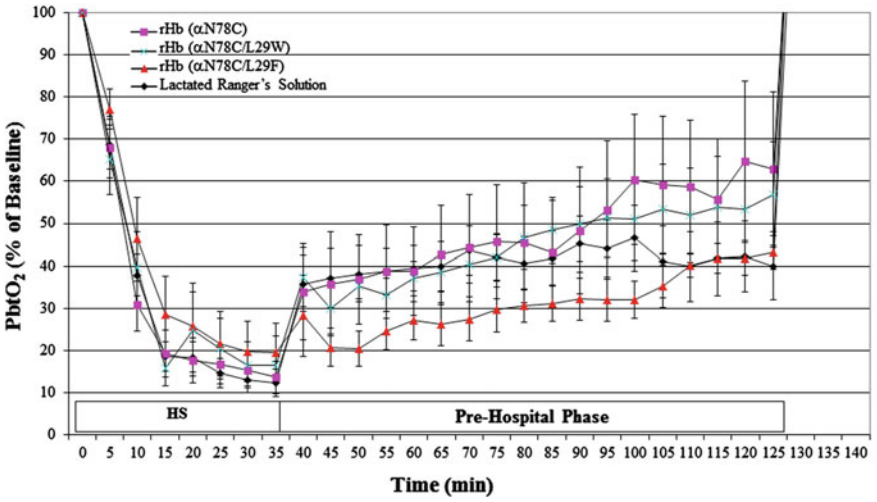


**Fig. 13.6** Brain tissue oxygen concentration ($PbtO_2$) during HS and Pre-Hosptital Phases on the ipsilateral side of controlled cortical impact induced traumatic brain injury in mice resuscitated with lactated Ringer solution, normal oxygen affinity rHb (αN78C), high oxygen affinity rHb (αN78C/L29F), or low oxygen affinity rHb (αN78C/L29W). Although there is a trend toward the lower $PbtO_2$ levels during resuscitation in the rHb (αN78C/L29F) group, this does not reach significance

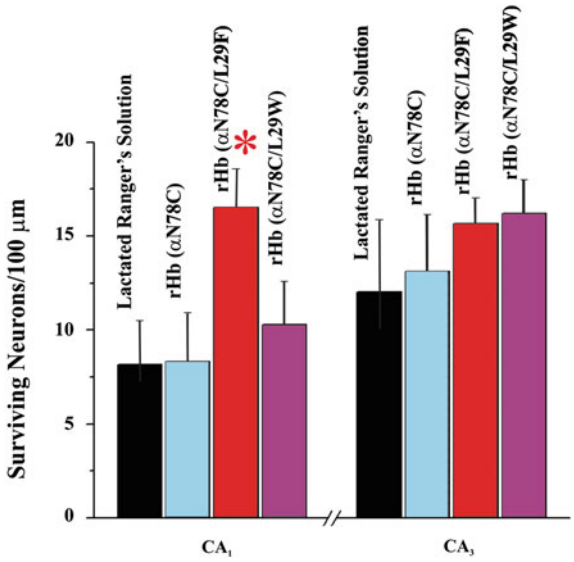


**Fig. 13.7** Surviving neurons in the CA1 and CA3 sectors of the hippocampus at 24 h after traumatic brain injury plus hemorrhagic shock in mice resuscitated with lactated Ringer solution, normal oxygen affinity rHb (αN78C), high oxygen affinity rHb (αN78C/L29F), or low oxygen affinity rHb (αN78C/L29W). In the CA1 region of the hippocampus, resuscitation with the rHb (αN78C/L29F) significantly increased neuronal survival (*$P < 0.05$ vs all other groups; ANOVA and Dunnett's test). In contrast, in the CA3 region of the hippocampus, there was no difference between groups on neuronal survival. Neuron densities in CA1 and CA3 regions were quantified in coronal brain sections taken through the dorsal hippocampus beneath the impact site and counts assessed per 100 µm length segments

However, neuronal counts in CA3 hippocampus do not differ significantly among treatment groups (Fig. 13.7). No neuronal loss was seen in the hippocampus contralateral to the impact (data not shown).

## 13.4 Discussion

The overall goal of this study is to develop high molecular weight hemoglobins with unique oxygen binding properties for use as resuscitation fluids. Development of an oxygen carrier of larger size can theoretically prolong the circulation time (Fronticelli et al. 2001; Chauvierre et al. 2010). To achieve this end, we have substituted αAsn78 on the surface of the tetrameric hemoglobin with cysteine to form an octameric structure that is stable in the presence of fresh human plasma (Brillet et al. 2012). The rHb (αN78C) is functionally similar to Hb A (Brillet et al. 2012) and our NMR studies here (Figs. 13.3 and 13.4) demonstrated that the heme pockets, the $\alpha_1\beta_1$ and the $\alpha_1\beta_2$ inter-subunit interfaces are also structurally similar to Hb A.

NO scavenging by extracellular HBOCs has been suggested to be the cause of hypertension and this effect can possibly be negated by employing Hbs with lower rates of NO-induced oxidation (Doherty et al. 1998). The leucine residue at B10 is part of the distal heme pocket of myoglobin and hemoglobin (Dickerson and Geis 1983). Previous studies have shown that mutation of Leu29 (the B10 residue) of myoglobin and the α-subunit of hemoglobin can reduce the rate of autoxidation and NO-induced oxidation (Wiltrout et al. 2005; Carver et al. 1992). Hence, we replaced αLeu29 on rHb (αN78C) with phenylalanine or tryptophan to generate hemoglobin octamers with B10 mutations.

rHb (αL29F) and rHb (αL29W) have been studied by our group (Wiltrout et al. 2005). rHb (αL29F) has high while rHb (αL29W) has low oxygen binding affinity ($P_{50}$). The octamers behave similarly, rHb (αN78C/L29F) mutant has high while the rHb (αN78C/L29W) has $P_{50}$ value (Fig. 13.2). Accompanying the change in oxygen binding affinity is a perturbation in the tertiary structure of the α-subunit heme pocket. The heme pocket of the $\beta$-subunit has not been disturbed (Figs. 13.3 and 13.4). Furthermore, these B10 mutations have not changed the subunit interfaces of the ligated hemoglobin (Fig 13.3). However, slight tertiary structural changes occur at the $\alpha_1\beta_2$ interface in the unligated (deoxy) B10 mutants (Fig. 13.4). Whether these structural changes in the mutants are related to the diminished $n_{50}$ values remain to be investigated.

These three octamers have been used as resuscitation solutions in our mouse model. This is the first application of octameric rHb in any clinically relevant *in vivo* model. We have chosen to study TBI combined with HS because this combined insult has emerged as a major combat casualty scenario in the United States Army due to improvised explosive devices (Bauman et al. 2009). Secondary insults also are common in civilian TBI and contribute to increases in morbidity and mortality (Chesnut et al. 1993). We have demonstrated that CCI (at the injury level used) or HS alone produces little damage to the CA1 and CA3 hippocampal regions, but injury and resultant neuronal death are significantly exacerbated when CCI and HS are combined (Dennis et al. 2009). In addition, this combined insult represents a setting where a cell-free Hb solution could have special potential, given the need to resuscitate with a small volume solution to limit brain edema, while attempting to optimize oxygen delivery to the highly vulnerable traumatically injured brain where metabolic demands are great (Hovda et al. 1995).

Our study with these novel rHbs demonstrated several interesting findings. First, rHbs, regardless of their oxygen affinity, restored systemic hemodynamics after TBI combined with HS and normalized markers of global tissue metabolism as suggested by systemic lactate and pH. This is in contrast to the failing resuscitation efforts that results from use of the standard LR. Even at 4 times the resuscitation volume as that of rHbs, LR does not improve MAP into the target range during the Pre-Hospital Phase. It is well known that TBI increases markedly the sensitivity to secondary hemorrhage and contributes to a refractory hypotension during conventional resuscitation (Yuan and Wade 1992). And our prior reports in murine models of TBI plus HS confirm that observation (Hemerka et al. 2012; Dennis et al. 2009).

A second interesting effect of octameric rHbs in this study is that the MAP profiles produced during resuscitation were predicted by the molecular structure of the rHbs. rHb (αN78C/L29F) and rHb (αN78C/L29W) have an amino acid substitute at the distal heme pocket (B10). *In vitro* experiments have concluded that rHbs carrying a single phenyalanine or tryptophan substitute at αLeu29 have reduced NO-induced oxidation rates (Eich et al. 1996; Olson et al. 1997; Wiltrout et al. 2005). The results imply that these mutants have diminished interaction with NO. We observed a supra-baseline hypertensive response after resuscitation only for rHb (αN78C) that has surface residue replacement. The other two octamers tested carry additional αLeu29 substitutions and they do not exhibit this hypertensive response (Fig. 13.5). Indeed, based on testing of numerous crystalloid, colloid, and Hb-based resuscitation fluids in our mouse model (Dennis et al. 2009; Exo et al. 2009; Shellington et al. 2011), rHb (αN78C) is the only agent that we have tested that has produced a hypertensive response. We recognize, however, that the effect of these octameric rHbs on 'vasoactivity' *in vivo* is likely to be complex and could involve mechanisms other than a simple change in the NO interacting site, such as differences in Hb-endothelial interaction or pre-mature arteriolar oxygen release, among other possibilities (Rohlfs et al. 1998). Nevertheless, given the general similarities of these octameric Hbs, we believe that the differences in molecular structure at αLeu29 are most likely the cause of this finding. This interesting finding suggests that specific rHb modifications can potentially translate to *in vivo* effects. Future studies should specifically address this interesting mechanistic question.

Although statistically significant differences in $PbtO_2$ ipsilateral to the injury have not been seen between groups resuscitated with octameric rHbs of varying oxygen affinities, there are trends that suggest that the differences in oxygen affinity might have played some role. Specifically, the high oxygen affinity rHb (αN78C/L29F) exhibited a trend toward the lowest $PbtO_2$ during the Pre-Hospital Phase—with values lower than the LR group. Our study represented an initial exploration of this concept, which certainly warrants additional studies. TBI is well recognized to produce heterogeneous injury and the local $PbtO_2$ can vary greatly. A much larger sample size and additional experiments would be needed to appropriately test the potential efficacy of rHbs in this regard. Indeed, the "ideal" design of an rHb in the treatment of TBI combined with HS or other conditions is an intriguing concept that certainly merits further investigation. For blood substitutes (but not necessarily resuscitation agents), the advantages and disadvantages of Hbs with higher or lower oxygen affinity have been extensively explored. Kunert et al. (1996) reported that the administration of allosteric effector that reduces Hb affinity for oxygen can increase tissue $PO_2$, but decrease arteriolar diameters and blood flow. Because of the vasoactivity associated with low oxygen affinity Hb, some researchers believe that Hbs with high oxygen affinity are more desirable. Rohlfs et al. (1998) found that Hb vasoactivity was closely linked to decreased oxygen affinity, but not to NO consumption rates. They hypothesized that Hbs with lower oxygen affinity may cause premature $O_2$ release in arterioles and triggering autoregulatory vasoconstriction. In support of this hypothesis, a

polymerized low oxygen affinity bovine Hb ($P_{50}$ of 54 mm Hg) reduced tissue oxygenation in a hamster cheek-pouch model (Cabrales et al. 2008; Tsai et al. 2006). Consistent with that hypothesis, the high oxygen affinity rHb was the only one that exhibited a neuroprotective effect–enhancing neuronal survival in CA1 hippocampus (Fig. 13.7). It should be noted, that the CA1 hippocampus is highly vulnerable to both ischemia and oxidative stress after TBI, and is a highly vulnerable target in the setting of TBI plus HS (Dennis et al. 2009). In contrast, many investigators have suggested that a low oxygen affinity Hb would be desirable to facilitate $O_2$ delivery in resuscitation. Surprisingly, there are few data to support this intuitive hypothesis. Watanabe et al. (2008) transplanted transgenic bone marrow cells that express an extremely low oxygen affinity Hb ($P_{50}$ of 99 mm Hg vs $P_{50}$ of 48 mm Hg for normal mouse) into mice with chronic heart failure and reported increased $O_2$ supply to skeletal muscles and improved treadmill performance. Similary, Huang et al. (2005) reported that transfusion of low oxygen affinity Hb (Presbeterian Hb) to septic mice can improve survival. As for our high and low oxygen affinity octamers, we have shown previously that substitution of αLeu29 with either phenyalanine or tryptophan would lower the NO-induced oxidation of the rHbs (Wiltrout et al. 2005). Hence, further experiments are needed before we can delineate the oxygen affinity from NO interacting effect in animal models.

The debate regarding optimal $O_2$ affinity for blood substitutes will continue. Unfortunately, our data do not yield a definitive answer with regard to resuscitation of injured brain. We have found that the high oxygen affinity rHb [rHb (αN78C/L29F)] has the lowest numerical $PbtO_2$ (Fig. 13.6) and the paradoxically best acute neuronal survivial. TBI combined with HS is a complex model and additional studies should be pursued in other models.

Accompanying the potential benefits of rHbs related to hemodynamics, $O_2$ delivery and their small resuscitation volume requirements are their potential neurotoxicity. This may be an important issue when Hb solutions are used in patients with TBI, where blood–brain barrier injury is well recognized. It is known that the amount of native Hb that leaks into brain parenchyma after TBI may be sufficient to cause direct neuronal injury (Hellal et al. 2004; Wang et al. 2002). We have seen improved neuropathology only in the high affinity group in spite of greatly improved hemodynamics in all of the rHb groups versus LR. One possibility is that direct neurotoxicity may play a role. A covalently modified bovine polynitroxylated pegylated Hb has shown neuroprotection in this TBI with HS model (Shellington et al. 2011). This modified Hb also exhibits surprising neuroprotection in primary neuronal culture rather than the neurotoxicity seen with the parent unmodified bovine Hb (Shellington et al. 2011). Direct neurotoxicity, thus, may contribute to our ability to show neuroprotection only in the high oxygen affinity rHb group. The potential of an optimized rHb or covalently modified rHb to show neuroprotection in TBI combined with HS also deserves further investigation. Other markers of neuroprotection, such as brain edema, intracranial pressure, and long-term histological and cognitive outcomes, should also be studied.

There are several concerns to this initial exploratory study with novel octameric rHbs. First, it would be of interest if native human Hb and mouse Hb solutions were also tested. However, our primary focus was to compare these three rHbs with different oxygen affinities. Secondly, there are concerns that endotoxin in the rHb solutions could be problematic (Okajima et al. 2005). The approach taken by our group in purifying these rHbs appears to have addressed this concern and endotoxin levels are acceptable and hypotension has not been seen with our rHb administration. Nevertheless, we cannot rule out unrecognized effects of endotoxin, cell free Hb, or differences in oxygen affinity on either mortality or neuropathology in our model. Our study focused on comparing oxygen affinity and was not powered to assess mortality. Although not significantly different, mortality was numerically greater at 24 h in the low affinity rHb (αN78C/L29W) group. Nonetheless, no appreciable Pre-Hospital mortality was seen with any of the rHbs. Third, the pharmacokinetics of octameric rHbs has not been studied previously. There is no assay available to determine readily the blood levels of the octamers. Our hemodynamic data suggest that MAP is maintained for at least ~2 h. Fourth, although we believe the most critical period of resuscitation was the Pre-Hospital Phase, administration of 100 % $O_2$ and shed blood in the Hospital Phase in all groups may have limited differences between groups. Finally, the study was not conducted in a randomized fashion.

In summary, octameric rHbs normalized MAP and blood lactate with much smaller than LR in mice suffered TBI combined with HS. In principle, the difference in NO reactivity rather than oxygen affinity of Hbs appears to account for their difference in hemodynamic effects. The high oxygen affinity rHb (αN78C/L29F) shows the lowest $PbtO_2$ during resuscitation and is the only rHb to confer acute neuroprotection assessed at 24 h after injury. The impact of these interesting new rHbs on resuscitation of TBI, HS, and their combination deserve further explorations.

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# Chapter 14
# Liposome-Encapsulated Hemoglobin as an Artificial Oxygen Carrier: Technological Features, Manufacturing and Issues for Practical Application

**Shinichi Kaneda, Takanobu Ishizuka, Hiroshi Goto and Hiroaki Kasukawa**

## 14.1 Introduction

The clinical application of AOCs as a substitute for RBC transfusions has long been anticipated to solve several problems associated with blood transfusions. Expected applications include the use of AOCs in the treatment of ischemic diseases by increasing oxygen delivery, and such potential applications of AOCs have been strong motivators for development. AOCs can be categorized into two major groups, namely perfluorochemicals (PFC) and hemoglobin-based products, and the latter can be further subdivided into either non-capsule-type hemoglobin or encapsulated hemoglobin. Because hemoglobin plays a major role in the oxygen delivery by RBCs, the use of hemoglobin instead of PFC as AOCs would be reasonable in order to achieve the efficient oxygen transportation seen in RBCs. Although great efforts have been put into the development of hemoglobin-based oxygen carriers (HBOC), it nevertheless still remains in the developmental stage. Some non-capsule-type hemoglobins were discontinued at the clinical trial stage (Squires 2002; Kim and Greenburg 2004; Winslow 2006), while problems of non-capsule-type hemoglobins have also been recently reported in the results of a meta-analysis of clinical studies (Natanson et al. 2008). Even though the concept of hemoglobin encapsulation within an artificial membrane has been extensively studied, its complicated manufacturing process and high production costs have all hindered the progress in its development. Despite this, we have been persistently developing encapsulated hemoglobins as a RBC substitute based on the idea that hemoglobins should be encapsulated in vesicles as they are in RBCs. We have developed a LEH and established a manufacturing process for it, and have also

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evaluated its fundamental physicochemical and biological properties (Takahashi 1995; Ogata 2000a, b; Kaneda et al. 2008). Herein, we discuss its technological features, its manufacturing process, physiochemical properties, stability, biological safety, fundamental efficacy as an AOC, and remaining issues concerning the development of LEH.

## 14.2 Structural and Technical Features of LEH

The structural and technical features of LEH are shown in Fig. 14.1. LEH is comprised of highly concentrated purified hemoglobin prepared from outdated human red blood cells (HRBC) and liposomal lipid ingredients. The high concentration of hemoglobin in the internal aqueous phase of the liposomes is similar to the concentration in HRBC. The hemoglobin also contains inositol hexaphosphate (IHP) as an allosteric effector, with the IHP being able to more stably modulate the oxygen ($O_2$) affinity of the hemoglobin than 2, 3-bisphosphoglycerate. The $O_2$ affinity of LEH, reflected by its $P_{50}O_2$ value, can be controlled over a wide range of about 10–50 mmHg as the IHP concentration is varied. The liposomes have mean diameters of about 200–250 nm and are made of hydrogenated soybean phosphatidylcholine (HSPC), cholesterol, and stearic acid (SA). It has been previously reported that liposomes with diameters over 200 nm tend to rapidly distribute to reticuloendothelial organs. On the other hand, liposomes with smaller diameters would be less efficient at encapsulating hemoglobin. Therefore, the diameter range of 200–250 nm strikes a balance between stability in the circulation and hemoglobin encapsulation efficiency. In addition, the surface of the liposomes is modified by PEG5000-DSPE [N-(monomethoxypolyethyleneglycolcarbamyl) distearoylphosphatidyl-ethanolamine] to further improve its stability in the blood circulation, by reducing the adherence of plasma proteins to the liposomal surface as well as reducing its recognition as a foreign body by the mononuclear phagocytic system (MPS).

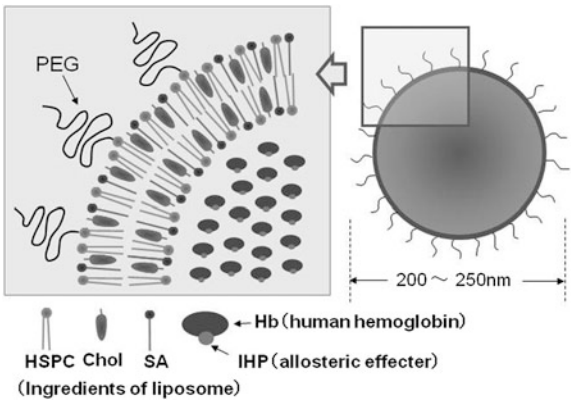


**Fig. 14.1** Structural and technical features of LEH. The LEH is composed of a lipid components, hydrogenated soybean phosphotidylcholin (HSPC), cholesterol, stearic acid (SA), and purified human hemoglobin with inositol hexaphosphate (IHP). The liposome surface is modified with PEG5000-DSPE

## 14.3 Physicochemical Properties of LEH

The typical physicochemical properties of LEH are shown in Table 14.1 (Kaneda et al. 2008). It shows the case of a LEH with lower oxygen affinity and high P50 value compared to HRBC. The low oxygen affinity of LEH would support higher oxygen transportation ability to the peripheral tissues, which means that the low oxygen affinity LEH (L-LEH) have oxygen transport efficiencies comparable to that of RBCs with relatively low hemoglobin concentration, particularly under conditions of high oxygen concentrations. On the other hand, high oxygen affinity LEH (H-LEH), with a low P50 value, show different oxygen delivery characteristics. It can deliver oxygen selectively to tissues with low oxygen tension such as ischemic tissues or organs. Furthermore, the small diameter of LEH lowers its viscosity and supports its property for preferential perfusion of the microcirculation. Taken together, these properties of LEH would contribute to its ability to oxygenate those ischemic tissues that are inadequately perfused.

## 14.4 Manufacturing Process of LEH

Figure 14.2 shows an outline of our manufacturing process for LEH. Purification of hemoglobin is one of the most important step in the manufacturing of LEH, because the characteristics of purified hemoglobin directly affect the function of LEH. To this end, we have established the following manufacturing process for hemoglobin purification. Initially, outdated HRBC obtained from the Japanese Red Cross Society were washed with sodium bicarbonate solution by centrifugation. Washed HRBC were hemolyzed and, at the same time, the process of virus inactivation was performed by the addition of a solvent and detergent (SD) that consisted of TNBP (tri (n-butyl) phosphate) and Triton X-100. Residual components of HRBC such as membrane fragments were removed by ultrafiltration, and virus removal was then performed by nanofiltration. Hemoglobin was subsequently concentrated using a reverse osmosis membrane and then sterilized with a sterilization filter. All of the hemoglobin purification processes were done under

**Table 14.1** Typical physicochemical properties of LEH

| Property | Value |
|---|---|
| Relative osmorality | 1 |
| pH | 7.5 |
| Hemoglobin (g/dL) | 6 |
| Total lipid (g/dL) | 4 |
| Hb/lipid (g/g) | 1.5 |
| Mean diameter (nm) | 230 |
| Methemoglobin (%) | <10 |
| $P_{50}O2$ (torr) | 40–50 |

low temperature conditions to prevent methemoglobin formation and hemoglobin denaturation. LEH was prepared by the following procedure. Purified human hemoglobin with added IHP was again sterilized with a sterilization filter, after which the hemoglobin were encapsulated by the lipid ingredients (all medicinal grade and pre-swollen with hot pure water) with a high speed emulsification apparatus. Large liposomal particles were then removed by two-step cross-flow filtration. The liposomes were subsequently deoxygenated by the addition of a sodium sulfite solution, after which external free hemoglobin was removed with exchange of outer solution of liposomes. A PEG5000-DSPE solution was then added and, liposomes were incubated under a temperature that is below the phase transition temperature of phosphatidylcholine. Once this is completed, liposomes were concentrated with cross-flow filtration and filled into plastic bags under aseptic and deoxygenated conditions. Manufactured LEH is hard to sterilize since over-sterilization by autoclaving will cause denaturation of hemoglobin and destabilization of the liposomal membrane. Furthermore, other sterilization methods, such as the use of gamma-rays and electron-rays, are also unfavorable for the sterilization of LEH because they can affect its physicochemical characteristics and stability. Here, we have constructed a manufacturing facility for the GMP production of LEH, where we are able to manufacture LEH under closed and fully aseptic conditions and use sterilized ingredients. All of the process lines for the

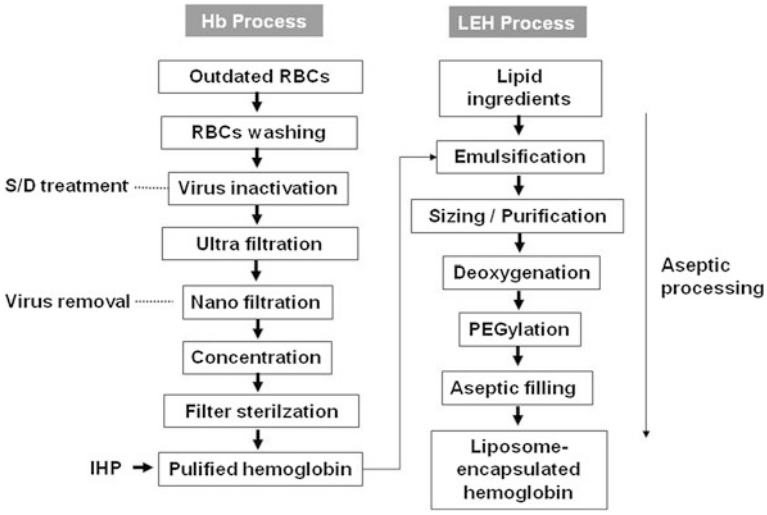


**Fig. 14.2** Manufacturing process of LEH. Purified human Hb solution is prepared from outdated human RBCs (HRBC). HRBC are washed with sodium bicarbonate solution by centrifugation. The RBCs are hemolyzed and treated by the SD method. The Hb is purified by ultrafiltration and then subjected to further nanofiltration as a virus removal step. The Hb is then concentrated and sterilized using a membrane filter. The purified Hb is encapsulated by the lipid ingredients using high-speed emulsification. External free hemoglobin and large-sized particles are removed by cross-flow filtration. Finally, the LEH is deoxygenated, its surface modified with PEG5000-DSPE, and then filled into plastic bags under aseptic conditions

manufacturing of LEH can be cleaned and sterilized by Cleaning in Place (CIP) and Sterilizing in Place (SIP), and can be adapted to automated, closed and aseptic manufacturing.

## 14.5 Stability of LEH

### *14.5.1 Stability During Storage*

The liposomes were stable during 12 months storage at temperatures between 2 and 8 °C, with no apparent changes being observed in the mean liposomal diameter and concentration of free hemoglobin in the outer solution (which would otherwise suggest hemoglobin leakage from the LEH), while the methemoglobin content decreased slightly during the observation period (Fig. 14.3 a–d) (Kaneda et al. 2008). This decrease in methemoglobin concentration was considered that reduction of methemoglobin occurred under deoxygenated conditions. However, there were no apparent alterations in the characteristics of LEH that would otherwise affect its function as an AOC, and stability is expected at least 12 months when properly stored.

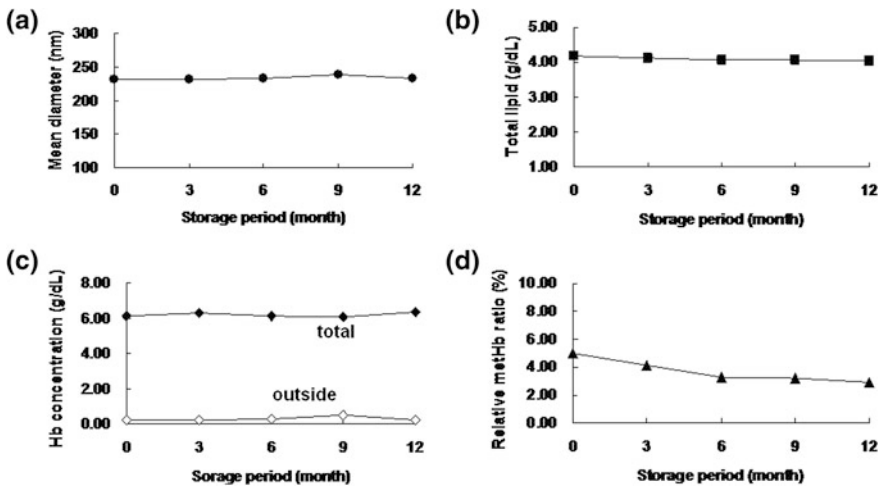


**Fig. 14.3** Stability of physicochemical properties during storage The diameter of liposomes was determined by the dynamic light scattering technique. Contents of lipid ingredients were measured by HPLC with IR detection. Hemoglobin concentration and methemoglobin content were measured by the cyanomethemoglobin method. **a** Mean diameter of liposomes, **b** Total lipid content, **c** Total Hb concentration and Hb concentration outside of liposomes, **d** Methemoglobin content

### *14.5.2 In vivo Stability*

We have also studied the in vivo stability of LEH. Pharmacokinetics studies were performed to clarify the kinetics of liposomes in the blood circulation as a marker of LEH stability. This is shown in Fig. 14.4 (Kaneda et al. 2008), where changing concentrations of human hemoglobin (as an indirect marker of LEH concentration) in the blood of rats and monkeys administered 20 mL/kg of LEH are displayed. There were apparent differences between rats and monkeys in their T1/2 and AUC in blood, i.e. the T1/2 of rats and monkeys were about 10 and 70 h, respectively. From the results of monkeys, however, longer retention times in human blood are anticipated. Large differences in the pharmacokinetics of other liposomal pharmaceuticals between some animal species have been reported, and this was thought to be mainly due to the differences in capacity of the RES. While modification of the liposomal surface with PEG is effective for stabilizing liposomes in the circulation, it cannot completely prevent the recognition of the liposome as a foreign body. The shorter circulating duration compared to RBCs and heavy burden placed on MPS by the rapid accumulation of liposomes still remain unsolved matters. Furthermore, since LEH do not have the methemoglobin reduction system that is normally present in RBCs, the issue of methemoglobin formation in LEH after administration into the blood is presently still unresolved. We have explored the possibility of reconstructing a methemoglobin reduction system within the LEH by the use of enzymes responsible for the systems in RBCs (Ogata et al. 1997). However, because of the advanced purification of hemoglobin that has recently been necessary to assure biological safety, implementing a methemoglobin reduction system has not been possible. Because of the generation of methemoglobin, the duration of oxygen transport efficiency of LEH is actually shorter than its long circulation time as a liposome. Therefore, the duration of

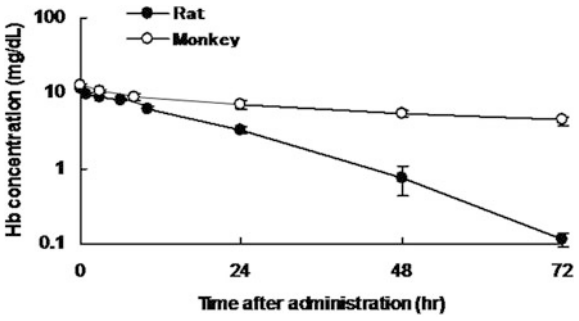


**Fig. 14.4** Differences in the concentrations of human hemoglobin in the blood of rats and monkeys. Male SD rats were administered 20 mL/kg of LEH via the tail vein, and the concentration of human hemoglobin in the rat's blood was determined by ELISA that specifically detects human hemoglobin. Male cynomolgus monkeys were also administered 20 mL/kg of LEH via the saphenous vein, and human hemoglobin in the monkey's blood was determined by the same method used for the rat

actual oxygen transport efficiency should be estimated by using the ratio between hemoglobin and methemoglobin concentrations of LEH in blood in order to understand the relations of oxygen supply by LEH to tissues and its efficacy as an AOC.

## 14.6 Safety as Pharmaceuticals

In single-dose toxicological studies in normal rats, lethality caused by congestion occurred at doses exceeding 100 mL/kg, which is comparable to commercially manufactured intravenous lipid emulsions. In repeated-dose toxicological studies in the therapeutic dose range in rats and monkeys, no serious adverse events were observed, but foam cells incorporating lipid particles were observed in reticulo-endothelial organs such as the liver and spleen. These pathological changes were clearly reversed by stopping LEH administration (Kaneda et al. 2008). In safety-pharmacological studies in dogs, transient decreases in the mean arterial pressure were observed just after starting LEH administration, but this reaction was not observed in the same types of studies in monkeys. As reported with other liposomal formulations, there are large inter-species differences for such cardiovascular responses (Szebeni et al. 2007). This response was found to be an acute phase reaction caused by the activation of the complement system and was dosing-rate dependent; decreasing the dosing rate reduced the intensity of the reaction. Based on the results of safety studies, LEH was thought to be fundamentally safe when used within the expected dose range of clinical applications. Furthermore, the inhibitory effects of LEH, Hb solution and HRBC on acetylcholine-induced vasodilation were compared and studied. Free hemoglobin is known to have a vasoconstrictive effect that is thought to be mainly due to NO trapping, and indeed some of the reported adverse effects of non-capsule-type hemoglobin may possibly be a result of this reaction (de Figueiredo 1998). As shown in Table 14.2, both L-LEH and H-LEH exhibit $IC_{50}$ values about ten fold higher than that of the Hb

**Table 14.2** Inhibitory effects on acetylcholine-induced vasodilation

| Sample | $IC_{50}$ (Hb concentration) | |
|---|---|---|
| | g/mL | nM |
| L-LEH | $3.08 \times 10^{-5}$ | 477.5 |
| H-LEH | $1.33 \times 10^{-5}$ | 206.2 |
| Hb | $4.63 \times 10^{-6}$ | 71.8 |
| HRBC | $1.30 \times 10^{-5}$ | 201.6 |

Isolated rat carotid artery rings were pre-contracted with $3 \times 10^{-7}$ mol/L phenylephrine in Krebs-Henseleit solution bubbling with a gas mixture consisting of 95 % $O_2$ + 5 % $CO_2$. $10^{-6}$ mol/L of acetylcholine was added to the organ bath, followed by the cumulative addition of L-LEH, H-LEH, Hb or HRBC. Inhibition rates were calculated as the percentages of maximum relaxation induced by acetylcholine. IC50 values were calculated by non-linear regression analysis

solution, and very similar to that of RBCs. These results were thought to demonstrate the superior safety of LEH as an AOC compared with non-capsule-type hemoglobin.

## 14.7 Fundamental Efficacy as an AOC

The basic efficacy of LEH as an AOC was evaluated in a rat model of massive hemorrhage. Administration of LEH dose-dependently decreased the plasma lactate (elevated by bleeding) to the same extent as washed HRBC, as shown in Fig. 14.5. The efficacy of LEH was also evaluated in monkeys that were highly hemodiluted. Compared with the saline treatment group, a tendency for longer survival was observed in the LEH treatment group. Mean arterial pressure returned to the baseline level in the LEH treatment group immediately after administration and was maintained throughout the observation periods, while the elevation of plasma lactate observed in the saline group tended to be suppressed in the LEH group (Fig. 14.6a, b) (Kaneda et al. 2008). These results suggest that the administered LEH demonstrated oxygen transport ability like RBCs and fundamental efficacy as an AOC.

## 14.8 Remaining Issues on Further Development

Regarding the function of LEH as mentioned previously, its short half-life in circulation is expected to be improved with advanced technologies as they emerge in the future. Furthermore, regulatory approval of the use of LEH involves complicated issues, since purified human hemoglobin is a biological product and

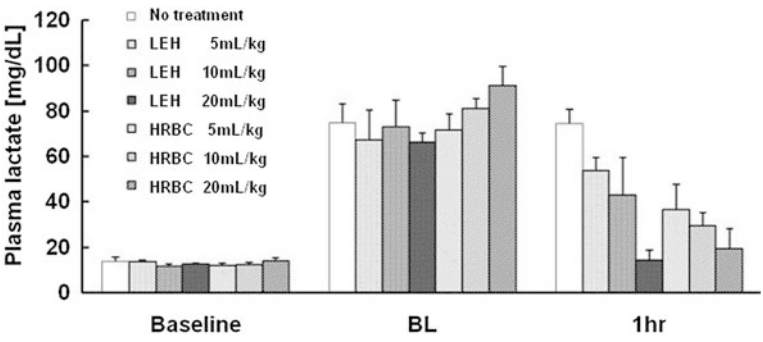


**Fig. 14.5** Efficacy of LEH in rats with massive hemorrhage. Anesthetized male SD rats were administered LEH or HRBC after isovolemic hemodilution and 20 mL/kg of bleeding. Baseline: before hemodilution and bleeding, BL: just after bleeding, 1hr:1 h after administration of LEH or HRBC

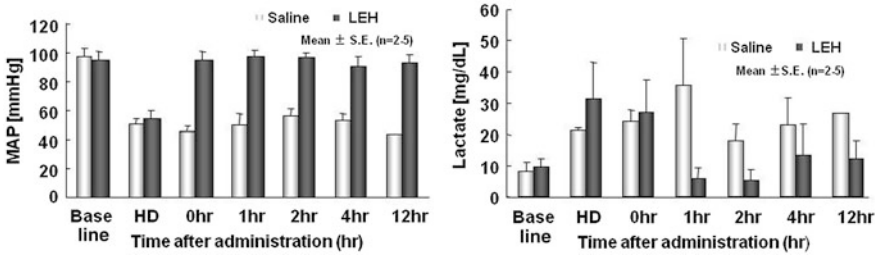


**Fig. 14.6** Efficacy of LEH in a model of severe hemodilution in the monkey. Cynomolgus monkeys were anesthetized with pentobarbital and then mechanically ventilated with 30 % oxygen to achieve a $PaO_2$ of about 150 mmHg. After baseline measurements, isovolemic hemodilution was done with a hydroxyethyl starch solution. This procedure was repeated until the hemoglobin concentration reached about 2 g/dL, following which LEH or Saline were administered and vital parameters measured for 12 h after resuscitation. **a** Mean arterial pressure, **b** Plasma lactate level

liposomes constitute a new drug formulation. We have developed LEH by considering the regulatory requirements for both biological products and new pharmaceuticals on quality assurance of LEH for approval in Japan. Further, we have referred the U.S. FDA draft guidance for blood substitutes (2004) as well as guidance from the Society of Blood Substitutes Japan regarding LEH (Takaori 2005). The GMP facility must be validated for its aseptic pharmaceutical manufacturing procedures and biological safety (such as virus clearance in the manufacturing process), which are basic but essential issues for the manufacturing of LEH as a new investigational drug. In addition, as previously mentioned, because LEH is difficult to sterilize after manufacture, assurance of aseptic manufacturing procedures is an utmost important matter during the production process; aseptic condition must be continuously maintained throughout LEH production and packaging. At the same time, these factors will impact on the cost of LEH manufacturing and are issues that need to be addressed in the future so that LEH can be made available at a reasonable cost.

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# Chapter 15
# Zero-Link Hemoglobin (OxyVita®): Impact of Molecular Design Characteristics on Pre-clinical Studies

**John P. Harrington and Hanna Wollocko**

## 15.1 Introduction and Background

For several decades extensive research efforts have been directed towards the molecular design of therapeutic oxygen carriers for use when whole blood or packed red blood cells are not available. (Winslow 2008; Alayash et al. 2007; Winslow 2007; Jahr et al. 2012). Most of these efforts have centered upon the development of cell-free (acellular) hemoglobin-based oxygen carriers (HBOC) with the expectation of adequate oxygen delivery as needed.

The rationale for considering the use of an acellular hemoglobin approach goes back many years and has its roots in nature as illustrated in both the terrestrial and marine environments (Hirsch and Harrington 2000; Harrington et al. 2007; Harington et al. 2010). Within natural evolution, the advantage of large polymeric acellular oxygen transport proteins was found to be effective for oxygen transport/delivery for many invertebrate organisms whether living within terrestrial or aquatic conditions (Royer et al. 2006 and 2007). The most obvious characteristic of these functioning natural polymers is the lack of a cellular membrane associated with mammalian red blood cell which normally houses a protective array of enzymes to maintain these hemoglobins in the reduced state necessary for functionality. Given these observations and our understanding of how these acellular oxygen delivery proteins function in vivo, the quest for a safe and efficacious acellular HBOC for clinical application has provided an opportunity to pursue a variety of approaches in molecular design enabling newer HBOCs to address the human physiological conditions(Buehler and Alayash 2008; Estep et al. 2008)

As one examines the efforts to address the development of a functioning HBOC for clinical use, there was the recognition that most of the proposed HBOCs need

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to function as an acellular molecular species within the human circulatory system. Unfortunately the details of how this was to occur did not receive sufficient attention in the earlier generation of HBOCs, resulting in a series of physiological effects that were recognized to be detrimental in many animal studies and for human clinical applications (Estep 2008).

The evolution of HBOC development at the molecular level has seen important design considerations incorporated into new molecular species with the goal of addressing the many negative characteristics associated with the first generation HBOCs (Stowell et al. 2001; Silverman and Weiskopf 2009; Alayash 2004). These design modifications were initiated with the introduction of an intermolecular cross-linking approach (DCL-Hb/HemAssist®, M.wt. 64 kDa, Baxter Corp., Deerfield. IL.) reducing dimerization of the initial tetrameric hemoglobin. This was followed by several different polymeric approaches involving intermolecular cross-linking polymerization by glutaraldehyde (a non-specific bifunctional cross-linking agent) to cross-link bovine hemoglobin tetramers producing a heterogeneous distribution of larger molecular weight species, (HBOC-201/Hemopure®, human application, and HBOC-200/Oxyglobin®, veterinary applications, M. wt. $>$ 150 kDa, OPK Biotech, Cambridge, MA), raffinose cross-linked human hemoglobin (Hb-Raffinose/Hemolink®, M.wt. $>$ 100 kDa), and pyridoxylated glutaraldehyde cross-linked human hemoglobin (Poly SFH-P PolyHeme®, M.wt. $>$ 120 kDa, Northfield Labs, Evanstone, IL.). Concurrently, several recombinant human hemoglobins were developed that allowed for modification of the oxygen binding properties of these recombinant hemoglobins (rHb1.0 rHb2.0, M.wt. 64 kDa, Somatogen/Baxter Corp.).

Eliminating the dimerization of stroma free hemoglobin (small molecular radii) associated with rapid elimination from the circulatory system and reduced stress within the glomerulus and kidneys was an immediate goal of this approach. Larger molecular weight cross-linked human and bovine hemoglobins were designed with the idea of increasing retention time within the circulation and limiting access to the smaller vascular membrane pores. The rationale here was to limit hemoglobin extravasation and mitigate nitric oxide (NO) binding that is now known to be involved with an increase in vasoconstriction coupled with an elevation in MAP (Olsen et al. 2004).

Pursuing a different direction to alter the molecular size and hydrodynamic properties of an HBOC, pegylated human Hb (MP4, Sangart Inc., San Diego, CA.) the Hemospan product (selected pegylation, M.wt. $\sim$ 90 kDa) was introduced in the 1990s to reduce extravasation thereby mitigating vasoconstriction and maintaining MAP (Vandegriff et al. 2003). This approach was associated with increased water of hydration of this HBOC leading to an increase in the effective hydrodynamic radius of these molecular species, reducing the tendency to extravasate.

Many of these early HBOCs evaluated in phase I−III studies received initial approval for clinical testing because they had demonstrated effectiveness in the delivery of oxygen in specific pre-clinical and specific clinical situations. However, observations of adverse events associated with their use prevented FDA regulatory approval for full clinical use (Winslow 2008; Alayash et al. 2007; Silverman and Weiskopf 2009). Several underlying reasons for this lack of

approval by the FDA were due to the inadequacy of the fundamental chemistry employed in the creation of several of these HBOCs and their physiochemical properties. Molecular size and shape, structural integrity (conformational integrity and quaternary structural intactness), redox behavior and stability (ability to be maintained and function in the reduced state) within the human circulatory system are vital to an HBOC functioning as a safe and efficacious therapeutic oxygen delivery system. Several earlier HBOC studies failed to provide this kind of structural information to the scientific community about the molecular integrity, stability, and redox activities of these HBOCs.

This chapter will provide a rationale for the development of a new generation HBOC, OxyVita Hb. Focus will be on its unique molecular design and the physiochemical properties associated with functionality essential for addressing many issues associated with previous attempts to produce an FDA approved therapeutic oxygen delivery acellular hemoglobin. The design and development of OxyVita Hb has resulted from understanding the lessons learned from the data and behavior, or lack thereof, of many previous pre-clinical and clinical studies carried out over the many years of work within this arena. The ultimate goal is to provide the clinical community with a new safe and efficacious therapeutic oxygen carrier as an alternative to blood transfusions when blood or red blood cells are not available. The original effort in the development of this new generation HBOC began in the laboratory of Professor Enrico Bucci and his co-workers (Razynska and Bucci 1998) at the University of Maryland, wherein they utilized a unique zero-linked hemoglobin polymerization technology. Further refinement of OxyVita's molecular properties has been carried out during a period of scale-up from laboratory preparation to a commercial scale level of production by OxyVita, Inc. It is now recognized that molecular size and the unique chemistry of OxyVita hemoglobin are directly linked to the success of initial pre-clinical studies conducted by many independent investigators throughout the United States (Matheson et al. 2002; Rebel et al. 2003; Mito et al. 2009; Reynolds et al. 2007; Jahr et al. 2008).

### *15.1.1 Development: A New Molecular Design Leading to the Production of the Liquid and Powder Forms of OxyVita Hemoglobin*

(a) Preparation and synthesis of OxyVita hemoglobin: Although any tetrameric mammalian hemoglobin may be used as the starting material for the preparation of a zero-linked polymeric hemoglobin, bovine blood was chosen as the raw material due to its ubiquitous availability world-wide. Fresh bovine blood is obtained from USDA-approved facilities, providing appropriate documentation of an animal herd. Purification of bovine hemoglobin is carried out through a process of red cell lysis using a hypotonic phosphate buffer, pH 7.4,

followed by a series of low-speed and high-speed centrifugation to remove cellular debris. The isolated tetrameric hemoglobins then undergo $\beta$-$\beta$ cross-linking [bis (3,5 dibromosalicyl-adipate)] in preparation for the synthesis of polymeric OxyVita Hb.

The zero-linked polymerization process is governed by the use of a chemical "activator" that initiates the production of intermolecular polymers. In the production of OxyVita Hb, the water soluble carboiimide, EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide], is responsible for the activation of the side chain carboxylate groups on the hemoglobin surface, resulting in a highly reactive and short-lived O-acylisourea derivative. This isourea by-product is very water soluble and is removed directly by dialysis. The complex is formed from the carboxylate groups of C-terminal and side chains of Glu and Asp globin residues of globin. These activated species react with the N-terminal amino groups or amino 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 (Grabarek and Gergely 1990). Some interference can occur during this activation process wherein the activated carboxylic groups may be hydrolyzed by water, limiting their reactivity with available lysyl amino groups. To improve on the efficiency of the polymerization process, a two-step approach to enhance the yield of the amide bond formation was introduced. The introduction of N-hydroxysulfosuccinimide (sulfo-NHS) to the carbodiimide reaction resulted in the formation of an intermediate sulfo-NHS ester, which then reacts with the amino groups (Staros and Wright 1986). One advantage of this approach is the ability to modulate the extent of polymerization by altering the relative amounts of sulfo-NHS and EDC within the reaction mixture. Control of the reaction rate, time and concentration within the polymerization process allows for better regulation of the average molecular weight sizes of an individual preparation.

A more extensive description of this zero-linked polymerization process as applied to the production of the original "zero-linked bovine hemoglobin" (ZL-HbBv) as first described and produced by Professor Enrico Bucci and co-workers can be found in Razynska and Bucci (1998). Earlier pre-clinical studies utilized these initial preparations which typically contained a heterogeneous distribution of high molecular weigh species in the range of 25 MDa (Bucci et al. 2007).

After the initial laboratory development of this bovine zero-linked polymeric hemoglobin, OxyVita, Inc. acquired the license for commercial manufacturing of this HBOC. It introduced some modifications of the preparation procedures in order to produce a more homogeneous molecular weight polymer with an average M. wt. of 17 MDa. Using anion-exchange (DEAE) and size exclusion chromatography (Fractogel 20−40), purification and isolation of discrete molecular weight fractions were achieved. Recent pre-clinical studies (Mito et al. 2009; Jahr et al. 2008; Jia and Alayash 2009) have used the OxyVita Hb preparations within their experimental protocols.

(b) Preparation of OxyVita hemoglobin-powder: The powder from of OxyVita Hb is produced by the lyophilization of the liquid form of this protein. Recent acquisition of a new lyophilization (Virtus, Inc) instrument allows for the production of this product under specific control processing. The new instrument has the capabilities of monitoring (CFR/211 compliant software) the essential steps associated within the freeze-drying process as well as automatically sealing of the product, thus reducing the chances of any endotoxin introduction during the course of the operation. In creating the powder form of OxyVita Hb consideration has been given to a number of different formulations that include the need for proper buffering, essential electrolytes and final osmolarity essential for infusion (IV) applications. Presently, re-constitution time (solubility) is between 10 and 30 s depending upon the compositional constituents.

## 15.2 Chemical and Structural Properties of OxyVita Hb

(a) Unique chemistry: The differentiating chemical and structural properties of OxyVita Hb are due fundamentally to the unique chemical and physical methods utilized in its production as described in the methods section. OxyVita Hb is synthesized through a polymerization reaction of purified ($\beta\beta$)-cross-linked tetrameric bovine hemoglobin using controlled activators which are removed after the initial phase of synthesis. This activation process primarily involves the carboxylic surface residues of the cross-linked tetramers which lead to the formation of the "zero-linked" polymeric molecular species. Selected modulation of these reactions allows for specific lysine residue involvement due to the differential pKs exhibited by these residues at pH 6.7. This approach allows for the absence of any chemical linkers between tetramers remaining within the product, eliminating possible side chain reaction concerns, such as reversibility and decomposition due to weak chemical bonds, dependency on temperature and pressure, and residual toxicity. The pseudopeptide bonds between the globin chains themselves provide a dramatic increase in the structural stability of OxyVita Hb as discussed in the following section (Harrington et al. 2010; Harrington et al. 2011).

The modified production methods allow for enhanced manufacturing and quality control resulting in sustained reproducible batch to batch preparations with a mean molecular weight of 17 MDa as determined by dynamic light scattering. Less than 5 % methemoglobin is present in each preparation. Given the unique chemistry associated with the use of reaction activators, variation of concentration of components involved, time and temperature of polymerization, this flexible polymerization process allows for the production of a range of molecular weight molecules, which may find different clinical applications

in the future. Another advantage is this polymerization process allows for the use of any mammalian blood (tetrameric hemoglobin) as the starting raw material for a zero-linked polymeric hemoglobin production.

(b) Implications for the structural-functional relationship of OxyVita Hb: These pseudo-peptide bonds created by the use of activiators, allow the tetrameric hemoglobin molecular linkages within OxyVita Hb to play a crucial role in the overall conformational stability of these large polymeric molecules. Essential structural integrity and biological functionality depend upon the integrity of intramolecular and intermolecular bonds within the tetrameric units and between these multiple tetrameric units ($\sim$1000 hemes/polymer) that go to form this "super-polymeric" HBOC. Given the inherent secondary and tertiary structure of bovine hemoglobin ($\sim$75 % $\alpha$-helical) and the fact that within each tetramer cross-linking between the $\beta$-82 lysines residues occurs providing inherent tetrameric stability as well, indicates OxyVita Hb will possess increased conformational stability and be very resistant to molecular unfolding. This resistance to conformational unfolding may provide increased protection to the heme-iron moieties responsible for the transport and reversibly binding of molecular oxygen within the circulatory system and oxygen delivery as needed.

Studies on the structural integrity of this "super-polymeric" HBOC carried out by isothermal unfolding studies at 37° C using urea as the perturbant of the secondary and tertiary structure of these large molecules (Harrington et al. 2010; Harrington et al. 2011) are consistent with the presence of strong intermolecular bonding between these tetramers. Figure 15.1 demonstrates the resistance of this HBOC protein to molecular unfolding as compared to several natural acellular polymeric hemoglobins found in the terrestrial and marine environments. The Soret spectral region (350−450 nm) was used to determine the extent of secondary and tertiary conformational changes associated with this HBOC's unfolding due to its extreme sensitivity to alterations within the heme environment. When tetrameric hemoglobin molecules are in the presence of a conformational perturbant, such as urea, increasing the urea's concentration leads to the disruption of many intramolecular interactions responsible for the maintenance of the integrity of the native functional structure.

Another advantage of using the Soret spectral region is that it allows for analysis of the redox state of the hemoglobin associated with the unfolding process. In the case of most hemoglobins, spectral wavelength shifts to lower wavelength (blue shift) are associated with an oxidation of the heme-iron as molecular unfolding occurs. Maintenance of the heme-iron complex in the reduced state (heme-$Fe^{+2}$) is essential for the reversible binding/release of molecular oxygen in vivo. In contrast to LtHb and ArHb, both large acellular natural polymeric hemoglobins, wherein the Soret wavelength maximum blue shifts are 413 → 400 nm and 412→ 398 nm, respectively, OxyVita Hb undergoes little change in the Soret maximum, 410 → 408 nm over the entire range of increasing urea concentrations (Fig. 15.2). This significant blue shift,

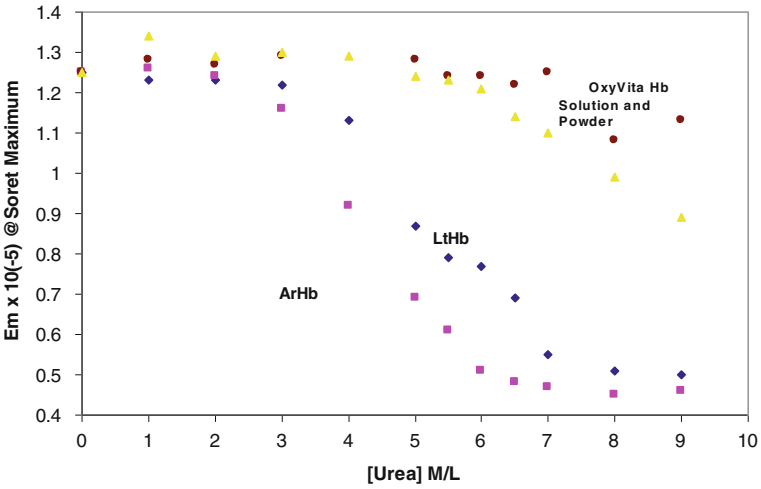


**Fig. 15.1** Isothermal unfolding of acellular hemoglobins (arenicola hemoglobin, ArHb; lumbricus hemoglobin, LtHb; and OxyVita Hb, liquid and powder preparations) in the presence of increasing concentrations of urea at T = 37° C. All solutions were equilibrated for 30 min prior to spectral runs (Harrington et al. 2010)

indicative of methemoglobin formation (heme-$Fe^{+3}$), is associated with an increase in the extent of heme exposure during subunit dissociation and unfolding within these natural acellular hemoglobins (Harrington et al. 2010). Methemoglobin formation leads to a decrease in oxygen-carrying capacity.

Acellular hemoglobins exhibit varying amount of methemoglobin formation via autoxidation with the potential for hemichrome formation and eventual release of the heme–iron which has been associated with cellular and tissue oxidative damage. Interestingly, a study by Jia and Alayash (Jia and Alayash 2009) clearly demonstrated that the zero-linked (OxyVita Hb) polymer gave no additional evidence of heme–iron loss compared to the initial bovine tetramers from which OxyVita Hb is produced. They suggested that its heme stability is related to a well-defined compact conformational structure of this large polymer. This is consistent with the observed resistance to the isothermal urea unfolding studies carried out as describe above which demonstrated the inherent structural stability of OxyVita Hb as well as its reduced tendency to undergo oxidation in the presence of the denaturing pertubant (Harington et al. 2010)).

A related observation on the redox behavior of OxyVita Hb is its ability to be reduced back to the oxyhemoglobin state, albeit slowly, in the presence of ascorbic acid, a known reducing agent often found in the human plasma. This reaction has the potential to offer protection to the OxyVita Hb within the circulatory system without the benefit of the normal protective reducing enzymes that function within the red blood cell. Keeping the acellular OxyVita

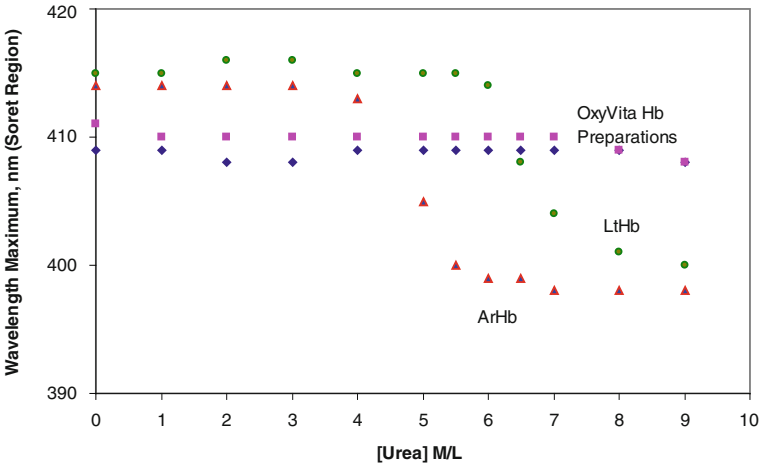


**Fig. 15.2** Changes in wavelength maximum within the Soret region (450−350 nm) for acellular hemoglobins in the presence of increasing concentrations of urea at T = 37° C (Harrington et al. 2010)

Hb in the reduced state will improve its functionality as an oxygen binding/release protein. Studies associated with the molecular events that may provide protection via other endogenous reducing agents for this acellular polymeric hemoglobin are on-going.

(c) OxyVita Hb's molecular size: Impact on other physiological functions: Table 15.1 presents the basic physicochemical properties of OxyVita Hb as determined by a wide range of biophysical and biochemical methods. We have successfully modified this polymeric hemoglobin (originally referred to a ZL-HbBv) through selected chemical steps to produce the "super-polymer" now referred to as OxyVita Hb possessing an average molecular weight of 17 MDa and a hydrodymanic radius of 360 Å. The relative hydrodynamic viscosity for a 6 g % solution of OxyVita Hb is similar to plasma (1.2−1.5 cP) and exhibits a colloidal osmotic pressure of 3 mm Hg (in lactated-Ringer's, pH 7.4, 23° C) approximately $1/10^{th}$ that of plasma. It exhibits a $P_{50} = 5-6$ mm Hg with an n value (Hill Coefficient) ~1. Less than 5 % methemoglobin is present in the final product.

Further studies using of a wide range of physical and chemical methods over the last several years have clearly shown that the polymeric hemoglobin constituting the OxyVita Hb solution, the OxyVita Hb powder form and the small volume resuscitation fluid (SVRF) are equivalent in all their properties as evident in Table 15.2.

(d) Impact of molecular design properties on pre-clinical studies: The unique chemistry associated with the chemical methods of synthesis of OxyVita Hb and the resultant molecular size and properties of this large polymeric hemoglobin described above have had a profound impact in addressing many

**Table 15.1** Physicochemical properties: OxyVita Hb (6 g %)

| Average hydrodynamic radius | =360 Å |
|---|---|
| Intrinsic viscosity $[\eta]_{c\to 0}$ | =4.2 ml/g |
| Average molecular weight | =17MDa |
| Colloidal oncotic pressure | =3 mmHg |
| Viscosity at 6 g/dl | =1.2−1.5 cP |
| Intravascular retention time | >10 h (cats, n = 5) |
| Oxygen affinity ($P_{50}$) | =5-6 mmHg |
| Cooperativity (hill, n) | =1.0−1.2 |
| Met Hb | <5 % |
| Stability-liquid $HbO_2$ | =21 days (Room T) |
| | =5 years (–80° C) |
| Stability-liquid HbCO | =1 year (Room T) |
| | >1 year (4° C)[a] |
| Stability-powder forms | ~5 years (Room T) |

Harrington et al. (2011)
[a] Presently still being stored refrigerated

**Table 15.2** Physicochemical properties of OxyVita Hb liquid and powder preparations

| | OxyVita solution | Small volume Resuscitation Fluid | OxyVita powder in water |
|---|---|---|---|
| Size exclusion LC-polymers | 100 % | 99.2 % | 100 % |
| Size exclusion LC-tetramers | 0 % | 0.8 % | 0 % |
| Spectral ratio $A_{576}/A_{540}$ | 0.963 | 0.963 | 0.964 |
| Autoxidation-MetHb | 3.7 % | 3.0 % | 7.92 % |
| Oxygen affinity $P^a_{50}$ | 5.95 | 5.26 | 6.59 |
| Hill coefficient (n value) | 1.1 | 1.2 | 1.2 |
| Dynamic light scattering[b] (average hydrodynamic radius) | 360 Å | 365 Å | 359 Å |
| Dynamic light scattering[b] (average molecular weight) | 17,337 kDa | 17,400 kDa | 17,154 kDa |
| pH | 7.46 | 7.50 | 7.58 |

[a] Hemox analzyer (T = 37° C, pH 7.50); [b] Dyna-Pro 801 (protein solution), Harrington et al. (2011)

fundamental issues within the field of HBOC development. The availability of OxyVita Hb has allowed many independent investigations to address and in several cases help to resolve these issues, which were identified by previous HBOC pre-clinical and clinical studies (Matheson et al. 2002; Rebel et al. 2003; Mito et al. 2009; Reynolds et al. 2007; Jahr et al. 2008). Concerns identified include but are not limited to: (1) loss of retention within the circulatory system; (2) extravasation and its impact on vasoconstriction coupled with increases in mean arterial pressure (MAP) upon HBOC infusion; (3) effect of HBOCs on the cerebral microcirculation and blood flow; (4) treatment of trauma and resuscitation responses; and (5) possible alterations

of coagulation behavior. During the last several years (Harrington and Wollocko 2010), each of these concerns has been investigated using OxyVita Hb or the earlier form of this HBOC (ZL-HbBv). These studies, along with the development of several other chemically modified HBOCs, have dramatically improved our understanding of many of the issues involved.

Retention, extravasation, vasocontriction concerns: Early on the question of loss of retention within the circulatory system associated with leakage of tetrameric or cross-linked tetrameric HBOCs was associated with stress on the glomerulus and kidneys. Recently the nature of acellular HBOC extravasation and its impact on vasoconstriction and observed increase in mean arterial blood pressure (MAP) have been linked to complex interactions with nitric oxide (NO). An improved understanding of these critical interactions has lead to a better appreciation of the role of molecular size and its impact on the binding of NO and the concomitant physiological changes (vasoconstriction) associated with any NO binding. The fact that increased molecular size can reduce the extent of acellular extravasation due to the molecules' inability to cross-over arterial or veinous membranes which exhibit various pore sizes (Matheson et al. 2000; Rippe and Haraldsson 1994) and bind with NO can lead to a reduction in vasoconstriction and help avoid BP elevation. In the case of OxyVita Hb, the larger radius of this molecule allows for greater retention time in the circulatory system with a half-life of 8−12 times longer that smaller radius HBOCs. All earlier animal studies carried out with OxyVita Hb have resulted in no observable vascular extravasation as determined by its absence in the renal hylar lymph after exchange transfusion experiments (Matheson et al. 2002). Furthermore, the recent work of Pittman and co-workers using a top-load rat model clearly showed that no vasoconstriction occurred in rat spinoitrapezius muscle using either form of OxyVita Hb (oxy or CO) and maintained tissue oxygenation (Song et al. 2012).

Cerebral microcirculation and blood flow behavior: The complex nature of the cerebral blood flow has been demonstrated by many investigations over the past decade (Scandinavian Stoke Study Group 1988; The Hemodilution in Stroke Study Group 1989). This complexity is even more apparent when attempts are made to understand and evaluate the role and effectiveness of HBOCs at delivering oxygen and reducing infarct volume during cerebral ischemia. In an detailed study using a mouse model, wherein the transfusion of large polymers of hemoglobin with a wide range on molecular sizes (ZLHb-Bv preparation) was carried out, it was determined that reduction (by 39 %) of the infarct volume was dependent on the concentration (6 g %) of this high-affinity hemoglobin ($P_{50} = 4$ mm Hg) and on the range of intermediate size hemoglobin polymers (500−14,000 kDa) transfused (Mito et al. 2009). Little or no reduction in the infract volume was observed under the following conditions: (1) transfusion in 5 % albumin solution; (2) a lower concentration (2−3 g %) of hemoglobin polymers of similar size; (3) hemoglobin polymers without removal of polymers <500 or >14,000 kDa; or (4) cross-linked hemoglobin tetramers with normal oxygen affinity (Mito et al. 2009).

The effective polymeric hemoglobin transfusion solution did not improve the distribution of cerebral blood flow during an ischemic event, nor did it alter blood flow to the brain or other major organs in the mouse model without ischemia. This latter finding also shows that these hemoglobin polymers do not initiate significant vasoconstriction in the brain or in peripheral vascular beds (Mito et al. 2009). Thus, in evaluating the potential of HBOCs for overcoming cerebral ischemia, a number of critical factors including, hemoglobin molecular size, concentration, and oxygen affinity, must be considered. Other HBOCs currently being evaluated may have the potential to address and further improve our understanding of the complex cerebral ischemia transfusion processes and protect the brain from ischemic strokes (Klaus et al. 2010).

Trauma treatment and resuscitation response: For the past several years, the assumption has been that early and rapid fluid resuscitation will restore blood pressure, reduce severe shock, and prevent multiple organ failure for treating uncontrolled hemorrhage. Recently, this approach has been challenged on the basis of new data indicating that aggressive attempts to normalize blood pressure with large fluid boluses produced increased bleeding, hemodynamic decompensation, and mortality (Dubick and Atkins 2003; Stern 2001). A newer procedure for permissive hypotensive resuscitation employing smaller volumes of hypertonic fluids is now favored small volume resuscitation fluid (SVRF). This approach has several advantages: (a) restoration of tissue perfusion accompanied by a modest increase in blood pressure with concomitant lowering of clotting factor dilution and re-bleeding; and (b) alleviation or reversal of lung damage. Within this approach, adequate blood pressure restoration needs to be sufficient without leading to re-bleeding, at the same time enabling adequate oxygen delivery to afford organ perfusion, thus reducing the possibility of multiple organ failure when full resuscitation is achieved.

This approach was recently tested in a Defense advanced research projects agency (DARPA) Reynolds et al. (2007). The specific objective was to determine a resuscitation strategy after 60 % hemorrhage in conscious male long-evans rats, which would lead to improved survival for 3 h in the absence of conventional large-volume crystalloid support. An additional component that was incorporated into this study was the use of OxyVita Hb in conjunction with a hypertonic saline solution and Hextend to enhance survival compared with standard small-volume resuscitation using Hextend only. The direct outcomes for this study included survival up to 3 h and maintenance of a MAP greater than 60 mmHg without additional fluid infusion (Sondeen et al. 2003). Test fluids administered included OxyVita Hb in a pressure-titrated infusion, Hextend titration, OxyVita Hb infused in a bolus method, and a Hextend bolus infusion. Throughout the course of these in vivo experiements, contstant monitoring of cardiovascular data, arterial gases, acid–base data, metabolites, electrolytes, Hb levels, and oxygen saturation were carried out. These occurred: (1) at baseline conditions, (2) at each 20 % hemorrhage increment, and (3) over 1−3 h after the initial hemorrhage. The most important finding from these studies was that a small-volume resuscitation treatment with OxyVita Hb significantly improved survival to 3 h and enhanced adequate MAP support for the

duration, independent of the method of administration. These results clearly support the idea that an OxyVita Hb-augmented hypertonic ‘‘cocktail’’ is an encouraging alternative to the standard method for improving the MAP support and survival (Reynolds et al. 2007).

Coagulation acitivity: Use of any HBOC as a transfusion medium whether in the context of an exchange fluid or a replacement fluid must not interfere with the coagulation processes normally found in whole blood. In several earlier studies it was reported that hetastarch solutions resulted in elevated coagulopathy. This effect appeared to be linked to an increase molecular weight via several different mechanisms (DeJonge and Levi 2001; Strauss et al. 2002; Huraux et al. 2001). Given these findings, the risk of coagulopathy in the application and use of high-molecular-weight HBOCs was investigated (Jahr et al. 2008). Using hemodilution during clinical resuscitation after hemorrhagic shock with different amounts of OxyVita Hb (6 g %), hetastarch, and oxyglobin (HBOC-200), coagulopathy behavior in 1:11 to 1:1 dilutions in whole blood was evaluated by a thromboelastographic (TEG) technique in real time. This method allows for direct analysis of clot strength and formation kinetics, and an indirect determination of platelet and coagulation factor functionality and availability (Royston and Von Kier 2001). These ex vivo TEG determinations used blood from healthy donors, eliminating interfering factors such as anticoagulants and other intravenous fluids. This enabled direct evaluation of the effect of the HBOC on the TEG results. Findings from this study (Jahr et al. 2008) indicated that both HBOCs had a hypocoagulative effect on coagulation, and that this was more pronounced than that of 6 % hetastarch at the highest levels of hemodilution. However, minimal coagulopathic effects are expected with the use of OxyVita Hb at the manufacturer’s expected maximum dose of 10 g or 2−3 ml/kg. Thus, some caution should be used when attempting to use OxyVita Hb as a large-volume oxygen plasma expander. Its application is not expected to lead to an increased risk of clinical bleeding when used in relatively small volumes (2−3 ml/kg).

An important related finding regarding the effect of an HBOC on coagulation is the presence of methemoglobin (Moallempour et al. 2009). A dose response study with HBOC-200 (Oxyglobin) was carried out using the thromboelastogram technique. The results from this investigation revealed that high concentrations of methemoglobin in HBOC-200 led to increased coagulation impairment. This negative effect may be because of the effects on platelet function and on other coagulatory proteins of concomitant oxidative species associated with increased methemoglobin levels. Platelets contain functionally important glycoprotein receptors containing thiol groups providing increased sites of interaction for these redox-sensitive structures (Essex and Li 2006). A significant characteristic of OxyVita Hb is the low methemoglobin levels associated with its preparation and its extraordinary resistance to molecular unfolding, which significantly reduces its conversion to methemoglobin in vitro and in vivo (Harrington et al. 2010; Harrington et al. 2011).

## 15.3 Summary

The focus of this chapter has been to address the reasons why OxyVita Hb, a new therapeutic oxygen carrier, is fundamentally different from previous generations of HBOCs. As our understanding of the relationship between the structural and functional behavior of the earlier HBOCs evolved, it became apparent that some of the structural characteristics incorporated into the modified HBOCs still resulted in problems of extravasation and vasoconstriction, oxidative degradation, and safety concerns when these HBOCs were evaluated within many pre-clinical and clinical studies. OxyVita Hb was designed to address these concerns. The fundamental aspects of OxyVita Hb's development and success to date within pre-clinical studies are based upon its unique chemical technology and the recognition that molecular size, hydrodynamic properties, negative surface charge (Harrington et al. 2011) and discrete functional properties all play important physiological roles as an HBOC. As described above, OxyVita Hb is differentiated from all other HBOCs presently being investigated due to: (1) its chemistry: a unique zero-linked polymerization method of synthesis; (2) well characterized physicochemical characteristics associated with its molecular properties and functional behavior; and (3) its potential for long-term storage (Table 15.1), due to these unique physiochemical properties, including extraordinary molecular stability, over a wide range of climatic conditions.

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# Chapter 16
# Polynitroxylated Pegylated Hemoglobin (PNPH): A Nanomedicine for Critical Care and Transfusion

**Li Ma, Frances M. Thompson, Dong Wang and Carleton J. C. Hsia**

## 16.1 Introduction

Evolving beyond the current generation of hemoglobin based oxygen carriers (HBOCs), a multifunctional hemoglobin nanomedicine (MHN) has been developed. PNPH is an example of a MHN, which contains one hemoglobin molecule per nanoparticle and functions from the intravascular plasma phase as a drug for small volume transfusion for the prevention or correction of interrupted or inadequate blood flow without oxidative stress in critical care and transfusion medicine (Hsia and Ma 2012, 2013).

Similar to HBOCs, PNPH retains the binding and transport capacity for oxygen, nitric oxide and carbon monoxide to function as a gas carrier in the plasma phase. However, in sharp contrast to current generation HBOCs, PNPH has the added functions of: (1) superoxide dismutase (SOD) mimetic activity, (2) catalase (CAT) mimetic activity, (3) unusual peroxidase activity, (4) nitric oxide protective activity by virtue of reduced superoxide through SOD activity, (5) hyperoncotic activity and (6) neuron protective activity. Thus PNPH has the added advantages of providing (1) improved hemodynamic stability, (2) superior volume expansion, (3) prevention of ischemia, (4) protection against reperfusion and inflammatory injuries and (5) hemoglobin detoxification capacity. In this chapter, data supporting the added functions are presented and PNPH is discussed as a drug for un-met medical needs such as traumatic brain injury complicated with

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hemorrhagic shock (TBI + HS), hemorrhagic and ischemic stroke, and sickle cell disease (SCD) as well as an alternative to red blood cell (RBC) transfusion to meet a worldwide unmet medical need.

## 16.2 PNPH

Through evolution, Hb as an oxygen carrier has diverged into two forms: a cellular Hb in vertebrates and an acellular Hb in invertebrates. Attempts to convert vertebrate cellular Hb into acellular Hb during the past two to three decades have failed to attenuate the intrinsic Hb toxicity or to counter the pro-oxidant activities of the vertebrate Hb. The significant challenges that had been encountered in HBOC development were highlighted at the FDA/NIH/HHS workshop in 2008 (FDA 2008). Following that workshop, the combination of challenges, negative perceptions, and difficulties in obtaining adequate funding for continued research and product evaluation led the NIH/FDA/DOD to convene a working group of experts in Boston in July 2011 to examine the future of oxygen therapeutics (NIH 2011). This later group re-affirmed the medical needs for oxygen therapeutics and outlined the basic and applied research needed to continue drug development for clinical applications including (1) a bridge to transfusion, (2) an alternative to blood transfusion, and (3) a therapeutic in indications where blood transfusion is not normally used.

Below we describe PNPH, a new generation product of hemoglobin based oxygen carrier (HBOC) that has evolved beyond the single function oxygen carriers to a MHN (Hsia and Ma 2012). We applied catalytic free-radical caged nitric oxide (cNO) technology to pegylated Hb to produce PNPH, a multifunctional, anti-oxidative nanomedicine that may also function as a life saving drug in massive volume transfusion.

### *16.2.1 HBOC Development*

Since the NIH/FDA/HHS 2008 Workshop, clinical trials of the current generation HBOCs have been terminated in the United States and continued clinical trials and commercialization have moved off shore. In countries where the AIDS epidemic and limited testing of banked blood makes the blood transfusion unsafe, the development and commercialization of current generation HBOCs may be justified. Additionally, use of an HBOC for delivery of carbon monoxide to treat SCD as an orphan indication is being pursued off shore. While these new approaches to current generation HBOCs seems to be making incremental improvements, they have not addressed the original limitations because these HBOCs still have the inherent toxicity of cell free Hb making them low therapeutic index oxygen carriers. However, if these off shore commercial uses and clinical trials are successful, it may aid in the approval and wide spread use of these HBOCs in the United States

and European markets as alternatives for transfusion of stored RBCs. The next generation HBOC may be dependent on development of invertebrate extracelluar hemoglobins, which lack the inherent toxicity of vertebrate cell free Hb making them higher therapeutic HBOCs (Elmer and Palmer 2012; Tsai et al. 2012).

### *16.2.2 MHN Development*

Our main focus has been development of drugs for the treatment of life threatening disruption of blood flow. Catalytic caged nitric oxide (cNO, aka nitroxide) platform technology has enabled the development of drugs that enhance blood flow in critical organs and treat the toxicity of acute Hb release, excessive superoxide production and nitric oxide depletion. Based on decades of research, we have developed a drug pipeline of cNO modified macromolecules, in particular albumin and Hb, as multifunctional neurovascular protective, hemodynamic stabilizing, antioxidative drugs for unmet medical needs in critical care medicine. From this pipeline, PNPH is being developed mainly to meet clinical indications where blood transfusion is not normally used. With PNPH, we are targeting major diseases like hemorrhagic and ischemic stroke, TBI + HS, and SCD. Herein we describe PNPH and how it may meet the challenges posted by the NIH/FDA/DOD Working Group of 2011.

### *16.2.3 PNPH - Science and Technology*

Focusing solely on the oxygen carrying and transport capacities of the HBOCs appears to have been a major flaw in the design of HBOCs. The conventional wisdom concerning the causes of the adverse effects of experimental HBOCs is that the primary mechanism of toxicity is nitric oxide (NO) scavenging at the endothelium, causing vasoconstriction and, paradoxically for a product intended to deliver oxygen, compromised tissue perfusion and oxygenation (Alayash 1999). Cell-free Hb derivatives tested to date may also bring another set of toxicities in addition to NO scavenging. These toxicities are related to pro-oxidant activity. While current generation HBOCs have attempted to deal with NO scavenging, they have not addressed the toxicities related to pro-oxidant Hb, which result from the combination of oxygen and heme iron in the absence of the controlling influence of the antioxidant enzymes of the red cell. The result can be the generation of toxic molecules such as superoxide, hydrogen peroxide, hydroxyl radical, oxoferryl porphyrin, etc. (Alayash 1999; Sloan 2003). Thus the reduction in oxygen delivery resulting from Hb'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 (Alayash 2004). In this view, cell-free Hb without appropriate regulation would actually add 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. Polynitroxylation may well provide the controlling influence to tame the pro-oxidant activity of Hb (Ma and Hsia 2005).

Polynitroxylation covalently links multiple nitroxides to various macromolecules. Nitroxides have been shown to act as mimics of SOD (Samuni et al. 1990; Krishna et al. 1992) and CAT (Krishna et al. 1996), inhibit peroxidation of lipids and lipoproteins (Damiani et al. 1994; Nilsson et al. 1989), and inhibit peroxynitrite-mediated nitration (Carroll et al. 2000). They are effective agents in the protection of cells against reactive oxygen species mediated damage caused by inflammation (Tsuhako et al. 2010; Cuzzocrea et al. 2004). More recently, their antihypertensive effects (Wilcox and Pearlman 2008), their vasodilatation effects (Simonsen et al. 2009), their neuroprotective effects in animal models of TBI (Deng-Bryant et al. 2008), and their protection of spinal cord mitochondria from oxidative stress (Xiong et al. 2009) have been reported. The therapeutic efficacy and mechanism of physiological effects of nitroxides has been comprehensively reviewed (Wilcox 2010). Unfortunately, the problem with low molecular weight nitroxides is that they show poor pharmacokinetics because they are isotropically distributed in vivo and rapidly bio-inactivated to their reduced hydroxylamine form with half-life on the order of minutes. The nitroxides on a macromolecule have distinct therapeutic advantage over low molecular weight nitroxides, such as 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol) (Rozantsev 1970) since linking multiple nitroxides to macromolecules has been shown to prolong the half-life of the nitroxide in vivo (Kuppusamy et al. 1996, 1998). Thus, polynitroxylation is a novel technology for improving the therapeutic index of nitroxides by linking multiple nitroxides to macromolecules for delivery to the vasculature.

Polynitroxylation maintains the oxygen carrying and delivery capabilities of Hb while adding multiple therapeutic activities such as vascular and multiple-organ protective activities against oxidative stress and reperfusion and inflammation injuries. The nitroxide and heme iron act in concert to perform antioxidant activities that mimic the behavior of the two physiologically important anti-oxidant enzymes that are found in red blood cells, SOD and CAT. Thus polynitroxylation technology restores the missing red cell enzymatic controls to the HBOC, thereby reducing superoxide concentration, $H_2O_2$ concentration, and NO depletion. The unusual peroxidase activity is also accomplished through further redox coupling of the nitroxide/heme iron complex with endogenous plasma antioxidants (Stoyanovsky et al. 2010).

PNPH derives its high therapeutic index from the synergy of the triple chemical modifications of carboxylation, pegylation, and polynitroxylation of 64Kd Hb. The 3D structure of PNPH is shown in Fig. 16.1 as a dissected view with a core Hb and its hydrated polyethylene glycol (Peg) shell, shown in light gray, formed by covalently attaching approximately 10 Peg molecules and an extra inner SOD mimetic nitroxide shell, shown in dark gray, formed by covalently attaching approximately 12 nitroxides (white balls). Figure 16.2 shows the electron paramagnetic resonance (EPR) spectrum of the free nitroxide compared to PNPH.

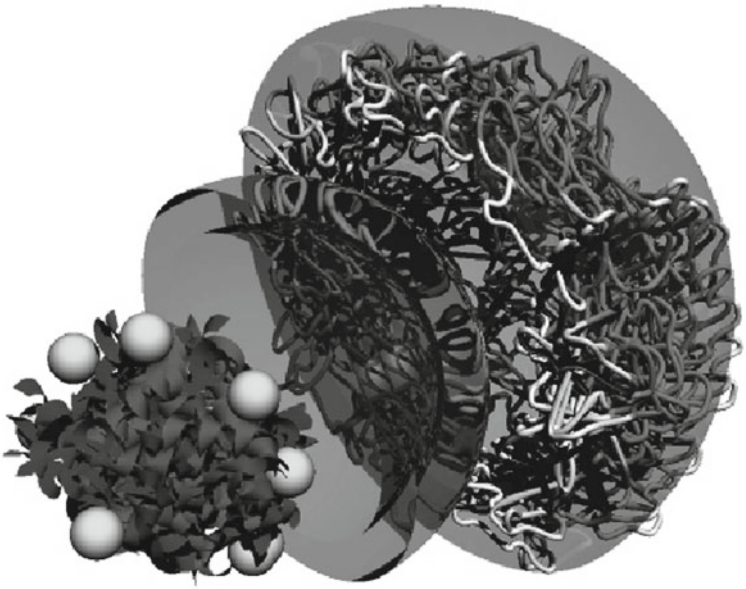

**Fig. 16.1** Structure of PNPH is shown in a dissected view with a core Hb and its outer hydrated Peg shell, shown in *light gray*, formed by covalently attaching approximately 10 Peg molecules and an extra inner SOD mimetic nitroxide shell, shown in *dark gray*, formed by covalently attaching approximately 12 nitroxides (*white* balls)

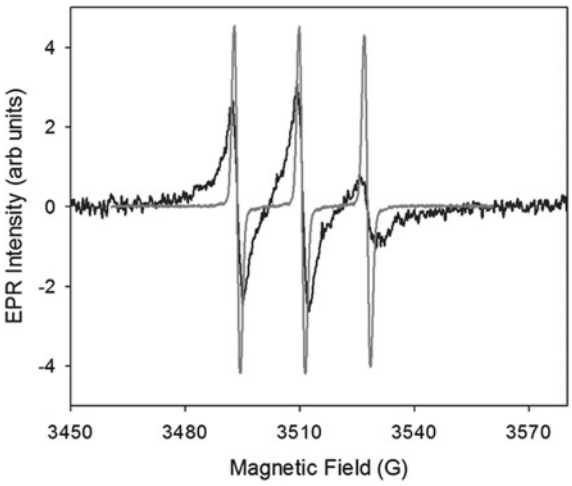


**Fig. 16.2** Electron Paramagnetic Spectroscopy (EPR) EPR spectra of BrAcTPO (*gray*) in alcohol at 0.1 μM and PNPH (*black*) at 1 μM. EPR conditions: microwave power, 0.5 mW; modulation amplitude, 0.5 G for BrACTPO and 2 G for PNPH; receiver gain, 36 dB; sweep width, 100 G for BrAcTPO and 150 G for PNPH; temperature, room temp

The spectrum of PNPH is characteristic of a covalent attachment of the nitroxides to the protein in which the nitroxides have a high spinning mobility independent of the protein and thus are freely moving around to form a nitroxide shell with SOD

mimetic activity. This SOD shell prevents the release of superoxide from the core Hb into the vascular space, which is the key to the success of PNPH as a multi-functional therapeutic drug that is completely free of Hb toxicities. Furthermore, this SOD shell is also able to break down excess superoxide in the vascular space. This prevention of superoxide release from the core Hb of PNPH and dismutation of vascular superoxide together lead to the correction of inadequate blood flow through conservation of vascular NO.

PNPH is a hyperoncotic colloid wherein nitroxides, with intrinsic SOD mimetic activity as described above, are covalently attached to pegylated carboxy Hb and are thereby also redox coupled with the heme iron to function as a CAT mimetic. Enclosed in a hydrated pegylation shell to reduce immunogenicity, these enzyme mimetic activities keep the Hb in an environment resembling that of the red blood cell to attenuate the toxicities observed with cell free Hb. PNPH has five structural and functional components contributing to its unique therapeutic activities: (1) Hb as the protein center provides oxygen carrier capability but is caboxylated to provide thermo stability and added anti-inflammatory activity, (2) the hydrated polyethylene glycol moieties of PNPH make it a hyperoncotic colloid (iso-oncotic at only 4 g/dl) which is important to stabilizing hemodynamics during hypotension and hypovolemia, (3) the nitroxide moieties of PNPH not only improve the safety of cell-free Hb but also provide anti-oxidant/anti-inflammatory, neuroprotective, and NO protective activities, (4) the 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 free plasma anti-oxidants such as ascorbate, which provides additional anti-oxidant activities.

These added intra-vascular and extra-vascular coupled redox reactions that attenuate vasoconstriction and the vascular inflammatory effects of ischemia and reperfusion are the new anti-oxidative stress therapeutic activities of this unique PNPH. *Thus this approach represents a paradigm shift in blood substitute development, away from the traditional focus on oxygen delivery and NO supplementation and toward a new focus on safely correcting inadequate blood flow and oxidative stress.*

## *16.2.4 PNPH Therapeutic Properties*

### 16.2.4.1 Enzyme Mimetic Activities

SOD and CAT mimetic activities: D'Agnillo and Chang (1998) reported that the complex formed by co-polymerization of Hb with endogenous red cell SOD and CAT released less free iron in the presence of $H_2O_2$ than Hb alone. Using the same assay method, free iron release was measured. The absorption measured at 562 nm was used for free iron release against standard calibration curve. The data shown in Fig. 16.3 demonstrates that PNPH released less free iron than pegylated bovine hemoglobin (PegHb) at different $H_2O_2$ concentrations.

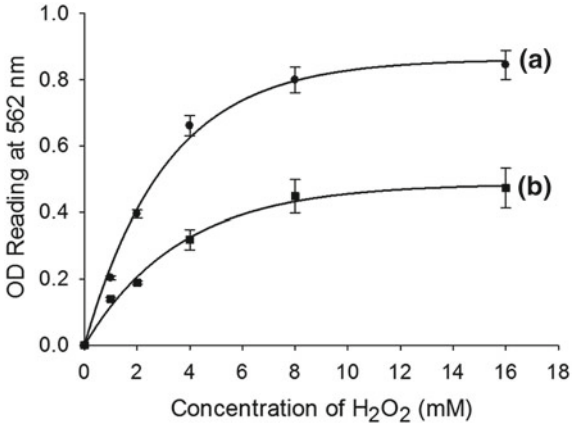


**Fig. 16.3** Comparison of free iron release from PNPH and PegHb under challenge by $H_2O_2$ Free iron was measured by the Ferrozine method (D'Agnillo and Chang 1998). A 2-ml reaction mixture of 10 μM hemoglobin tetramer of PEGHb (**a**) or PNPH (**b**) with various concentrations of $H_2O_2$ was mixed with 100 μL of 100 % trichloroacetic (TCA) acid and then titrated with ascorbic acid to final concentration of 0.01 %. Finally, 1 ml of ammonium acetate (10 %) and 0.3 ml of Ferrozine solution was added to complete the reaction. The absorption measured at 562 nm was used for free iron release (n = 3)

SOD mimetic activity: Levels of superoxide formation measured by luminol activity assays in the lung samples from WT littermate (WT control), and sickle cell mice (SS) exposed to saline (SS + NaCl), stroma free hemoglobin (SS + SFH), and PNPH (SS + PNPH) are shown in Fig. 16.4. Lung samples from SS mice exposed to saline had a significantly higher amount of superoxide

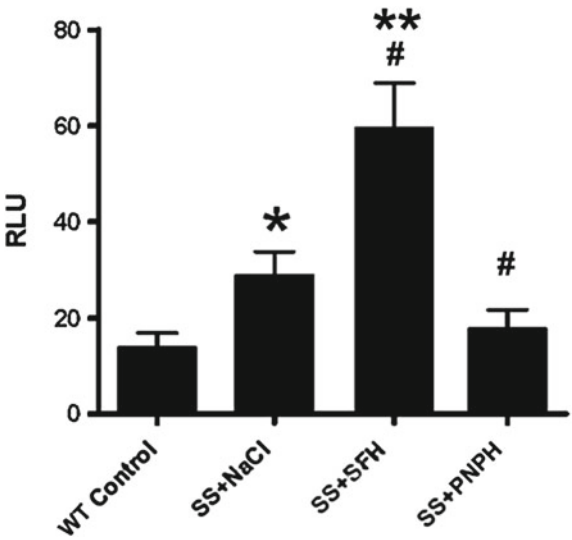


**Fig. 16.4** PNPH attenuation of superoxide formation in sickle cell (HbSS) mice. Luminol-enhanced chemiluminescence (CalBiochem, CA) was measured in relative light units (RLU) in lung samples of WT littermate (WT control), and sickle cell mice (SS) exposed to saline (SS + NaCl), stroma free hemoglobin (SS + SFH), and PNPH (SS + PNPH) (n = 4–7).* $p < 0.05$ and ** $p < 0.01$ compared with WT Control group # $p < 0.05$ omcpared with SS + NaCl group

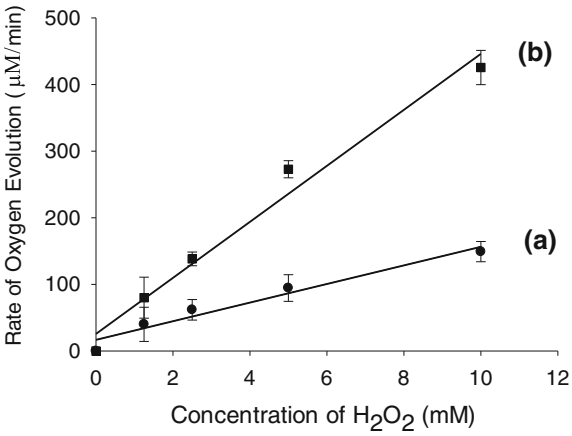


**Fig. 16.5** Comparison of the rate of oxygen evolution by PNPH and PegHb under challenge by hydrogen peroxide. Catalase mimetic activity of PegHb (**a**) or PNPH (**b**) was determined by their rate of oxygen evolution measured by oxygen electrode under challenge by hydrogen peroxide (Krishna et al. 1996). The reaction mixture contained 100 μM hemoglobin tetramer with various concentrations of $H_2O_2$ in 50 mM phosphate buffer, pH 7.4 at room temperature. The electrode was calibrated by air saturated phosphate buffer (n = 3)

formation when compared to WT control (* $P < 0.05$). Furthermore, stroma free hemoglobin resulted in a marked increase in superoxide formation as compared to SS + NaCl mice (# $P < 0.05$). In contrast, exposure to an equal amount of hemoglobin in the form of PNPH actually attenuated the increase in superoxide formation found in SS + NaCl mice (# $P < 0.05$) and had no significant difference compared with WT control mice. These in vivo findings suggested that PNPH reversed the elevation of superoxide formation and normalized the ROS signaling in the lung of the SS mice. (# $P < 0.05$) (Hsia and Ma 2009).

CAT mimetic activity: Inside the red cell, CAT converts $H_2O_2$ to $O_2$ and $H_2O$. Using the rate of oxygen evolution as a measure of CAT mimetic activity as previously described (Krishna 1994), the CAT mimetic activity of PNPH and PegHb were determined after adding various concentrations of $H_2O_2$. Data showed PNPH has a higher rate of oxygen release than that of PegHb at each $H_2O_2$ concentration (Fig. 16.5). PNPH also reduces the oxo-ferrylformation with exposure to $H_2O_2$. Data showed that, over time, $Fe^{4+}$ formation, measured by absorption at 545 nm, is much smaller in PNPH than in PegHb (Fig. 16.6).

#### 16.2.4.2 Neuroprotective Effect Against Hb Toxicity

Using primary cortical neuron cultures, data shows that PNPH is not only safe but also neuroprotective in three models; (a) a cytotoxicity study by incubating neurons with SFH or PNPH for 24 h, (b) study of PNPH protection against excitotoxicity in

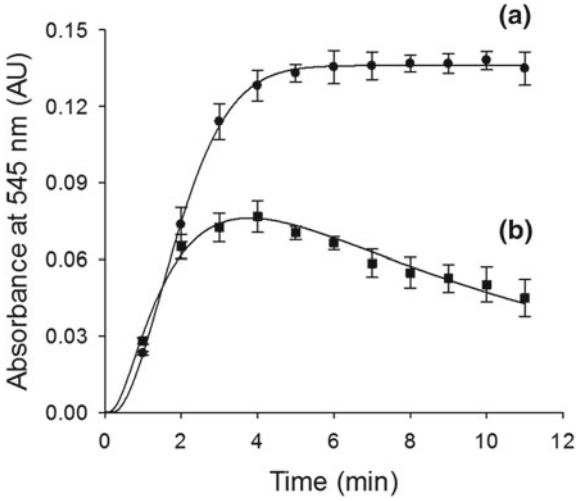


**Fig. 16.6** Comparison of oxo-ferryl formation by PNPH and PegHb with exposure to hydrogen peroxide. Oxo-ferryl formation of PegHb (**a**) and PNPH (**b**) with exposure to $H_2O_2$ was determined by their OD reading at 545 nm and is time dependent. The reaction mixtures contained 50 mM sodium phosphate buffer, pH 7.4, 25 μM $H_2O_2$, and 50 μM of either PegHb or PNPH

a glutamate/glycine-induced neuronal death model, and (c) study of PNPH protection against TBI-like stretch injury. Unlike SFH, which was neurotoxic, there was a lack of neurotoxicity of PNPH across a wide range of concentrations as measured by lactate dehydrogenase release and MTT viability tests. Also, dose dependent neuroprotection by PNPH in both glutamate/glycine induced neurotoxicity and neuronal stretch injury was demonstrated: when compared to saline treatment neuronal death was reduced between 40 and 85 % with PNPH treatment at concentrations of 0.2 and 2 μM in both tests. (Shellington DK et al. 2011).

#### 16.2.4.3 Vascular Protective Effect

By virtue of its SOD, CAT and peroxidase mimetic activities, PNPH is likely to stabilize the vasculature during ischemia and reduce hemorrhagic transformation arising from delayed reperfusion. Polynitroxylated albumin (PNA; VACNO®) has been shown to be protective of the vasculature by inhibiting hemorrhagic transformation in studies of delayed tissue type plasminogen activator (tPA) treatment (Hsia 2012). Similar studies are ongoing for PNPH.

#### 16.2.4.4 Physical Properties

The physical properties of PNPH are shown in Table 16.1.

**Table 16.1** Characteristics of PNPH

| | |
|---|---|
| Hb concentration | 4 g/dl |
| pH | 7–8 |
| Nitroxides per Hb molecule | ~ 12 |
| $P_{50}$ | 10 mmHg |
| Average molecular weight | 120 kDa |
| Hb = or < 64 kDa | <2 % |
| Viscosity | 3–4 |
| Colloid osmotic pressure | ~ 45 mmHg |

## *16.2.5 PNPH Clinical Indications*

### 16.2.5.1 Traumatic Brain Injury (TBI) Complicated by Hemorrhagic Shock (HS)

TBI is responsible for the greatest number of potential years of life lost from any cause and carries the highest burden on loss of quality-adjusted life-years among survivors (Gross et al. 1999). The primary injury to the brain occurs at the time of impact; however, subsequent compromise of cerebral perfusion can lead to an ischemic insult that extends the primary injury, creating a secondary brain injury (Stahel et al. 2008). The combination of TBI plus secondary insults such as HS can be devastating and new therapies are badly needed.

PNPH has been shown to be an efficacious, anti-oxidative hyper-colloid neuroprotective small volume resuscitative fluid for TBI + HS when compared to standard therapies in a model simulating the pre-hospital setting (Shellington et al. 2011). The data demonstrate that the pathophysiology from TBI + HS can be minimized with PNPH as a pre-hospital treatment and the exacerbation of edema caused by the large volume resuscitation with current standard care can be avoided (Brockman et al. 2012). PNPH requires the least volume to restore and maintain mean arterial blood pressure when compared to Lactated Ringer's (LR), standard civilian therapy, or Hextend (HEX), 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 region 1 of the hippocampus proper vs. HEX and LR. PNPH also reduced the exacerbation of edema caused by the large volume resuscitation of current standard care while maintaining cerebral oxygenation better than LR as measured by implanted direct oxygen electrodes. In addition, recent data suggest a substantive attenuation of the development of intracranial hypertension during resuscitation compared to conventional therapy in TBI plus severe HS in mice (Brockman et al. 2012). In a separate publication studying TBI + HS, in vivo measurement of hemodynamic response, cerebral perfusion and intracranial pressure (ICP) demonstrated a beneficial synergistic effect of breathing 100 % oxygen and PNPH resuscitation, which included reduced ICP (Blasiole et al. 2010). Thus, multifunctional PNPH is an exciting therapy for TBI resuscitation in the setting of hemorrhagic hypotension as it confers multiple benefits including functioning as a small volume resuscitation solution, more promptly

restoring MAP and CPP, reducing ICP, brain edema, and neuronal death when compared to standard resuscitation with LR. In on-going studies in the laboratories of Patrick Kochanek and his collaborators at the Safar Center for Resuscitation Research, PNPH also appears to have potential to confer benefit on some aspects of long-term cognitive function vs. resuscitation with LR, and certainly does not have deleterious effects on this very important clinical parameter.

#### 16.2.5.2 Stroke

"Time is brain" is the mantra for stroke treatment, and a drug safe for immediate (pre-hospital) and universal use in all stroke victims without the need for neuro-imaging would be a major breakthrough and a paradigm change in stroke therapy. The efficacy and safety requirements of such an early treatment drug is that it can enhance or maintain cerebral blood flow for oxygen delivery to the ischemic region without reperfusion damage and inflammation injuries and can also reduce the cerebral injuries from ongoing hemolysis in hemorrhagic stroke and hemorrhagic transformation in ischemic stroke. PNPH has shown such properties and, as discussed above, it has also been shown to protect neurons from Hb toxicity.

Transfusion of PNPH has been shown to be protective in a rat filament model of 2 h of middle cerebral artery occlusion (MCAO). Transfusion of 10 ml/kg of PNPH at 20 min of MCAO reduced the median infarct volume in the cerebral cortex from 40 % (37–47 % interquartile range; $n = 10$) in controls to 3 % (0–7 %; $n = 10$; $P < 0.001$) and in striatum from 78 % (66–88 %) to 34 % (0–37 %; $P < 0.001$). To determine whether delaying PNPH transfusion until 90 min of MCAO would improve penumbral perfusion, laser-Doppler flow (LDF) was measured in the ischemic border region where the reduction in LDF was less severe than in the core. LDF significantly increased from $48 \pm 18$ % of the pre-ischemic baseline to $67 \pm 21$ % ($P < 0.005$). Thus, PNPH transfusion has a significant therapeutic window for protection from transient MCAO and may act, in part, by stabilizing vascular function and improving collateral blood flow (Zhang 2013). The efficacy of PNPH to enhance blood flow in the ischemic penumbra while protecting the vasculature and reducing hemorrhagic transformation could expand the number of ischemic stroke patients treatable with lytic therapy well beyond the current 2–3 % per 2011 STAIR report (Albers 2011).

#### 16.2.5.3 Sickle Cell Disease

Daily administration of oral hydroxyurea is the first pharmacological intervention documented to provide clinically significant prevention of complications in sickle cell disease. Hydroxyurea treatment has recently been shown to reduce pain events, hospital admissions and the need for blood transfusions by 50 % and mortality by 40 %. However, there are side effects and patient's CBCs must be monitored every two weeks for evidence of bone marrow suppression. Once a stable or maximally tolerated dose is obtained, the patient can be monitored monthly. The long-term benefits and toxicities of hydroxyurea are unknown.

Transfusion of PNPH has recently been shown to significantly reduce the global insufficiency of vascular NO in transgenic SCD mice as measured by pulmonary hypertension and aortic stiffness. PNPH acts via the elevation of vascular NO through the suppression of the superoxide induced by cell free hemoglobin from the chronic hemolysis of SCD (Hsia and Ma 2009). Thus PNPH may represent an alternative to hydroxyurea SCD therapy or increase efficacy of hydroxyurea treatment with conjunctive use.

#### 16.2.5.4 Alternative to Stored Red Blood Cells

Massive transfusion of blood can lead to clinical complications, including multi-organ dysfunction and death. These severe clinical outcomes have been associated with the use of RBCs stored for longer periods of time in a recent meta-analysis on available data including 409,966 patients and 21 studies (Wang et al. 2012). In a guinea pig transfusion model with blood stored under standard blood banking conditions for 2 (new), 21 (intermediate), or 28 days (old blood), transfusion with old but not new blood led to intravascular hemolysis, acute hypertension, vascular injury, and kidney dysfunction associated with the pathophysiology driven by hemolysis of old blood (Baek et al. 2012).

The most effective and safest way to avoid the problem of RBC aging with storage is simply to decrease the storage time, for example from 42 to 21 days or even less. But such a decrease in the allowed storage time for RBCs will cause a dramatic decrease in the blood supply all over the world, especially in the developed countries like US that have a much higher stable number of blood donors than the developing countries, which are already struggling with blood supply shortage. When this happens, it will be crucial that safe alternatives to RBC transfusion are available.

Additional adverse effects of blood transfusion relate to the antigenicity of donor blood and its ability to transmit infections. In addition to being a potentially high therapeutic index drug, PNPH has pegylation to reduce antigenicity and eliminates, or at least substantially reduces, the incidents of disease transmission. Hence, PNPH may be an ideal alternative to RBC transfusion and could play a major role in the setting of trauma care and some elective surgeries and could also benefit patients with medical conditions who are in need of long-term blood transfusions and experience immunological sensitization.

### *16.2.6 PNPH- Summary of Pre-clinical, Current Status, and Future Clinical Strategy*

In additional to the efficacy testing in the various indications discussed above, a preliminary toxicology study in rats has been completed. The study concluded that there was no mortality or clinical observations that were associated with systemic

toxicity in the rats. Weight gain and food consumption was indicative of good overall health of the rats.

Based on this preliminary toxicology study and the results of the multiple preclinical efficacy studies, the FDA concurred with SynZyme on November 1, 2012, at a PIND meeting that the development of PNPH as a therapeutic for critical care and transfusion medicine was justified. NINDS funding is expected to continue to IND submission in 2016 through NINDS grant #U44 NS07032. Private funding through corporate partnering could shorten the timeline. Looking several years into the future, the clinical development strategy is expected to be to establish the safety of PNPH as a small volume resuscitative fluid in Phase I and then expand to multiple exploratory Phase II clinical trials in the indications discussed above where efficacy has been established. Proposed endpoints for these exploratory Phase II clinical trials could be:

- TBI + HS: Superior 6 month neurological outcome or survival with PNPH over standard care to benefit the more than 1.4 million TBI patients per year in America.
- Stroke: Extension of the tPA treatment window with early PNPH treatment. There are over 700,000 ischemic stroke patients annually, yet only 2–3 % are treated with tPA after 16 years of continuous clinical use in America.
- SCD: Reduction of pain events, hospital admissions and the need for blood transfusions by >50 % and mortality by >40 % when alone or in conjunctive treatments with Hydrea to benefit the ~100,000 American SCD patients.
- Alternative to stored RBCs: Compare blood flow response and biomarkers between fresh RBCs, aged RBCs, and PNPH to reduce the over 15 million units of stored RBCs being transfused in the U.S. annually.

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# Chapter 17
# ATP-Adenosine-GSH Crosslinked Hb, An Oxygen Carrier with Pharmacological Properties for Multiple Therapeutic Indications

**Jan Simoni, Grace Simoni and John F. Moeller**

The quest to develop a viable substitute for human blood has been ongoing for many decades. Many attempts to create an effective blood substitute fall short because of toxicity issues and at present no product is commercially available. Armed with an awareness of the problems associated with the performance of the first generation blood substitute products, which are linked to hemoglobin's (Hb) intrinsic toxicity that causes hypertension, oxidative and inflammatory reactions, gastrointestinal disorders and even heart attack and stroke, researchers at Texas Tech University Health Sciences Center created a novel concept of pharmacological cross-linking of Hb with ATP, adenosine and reduced glutathione (GSH) that resulted in the non-toxic and effective ATP-ADO-GSH-Hb product. This novel blood substitute possesses vasodilatory, anti-oxidant, anti-inflammatory and erythropoietic properties; all of which are beneficial to the patients suffering from acute and chronic anemias, sickle cell disease, ischemic diseases including heart attack and stroke, and cancer. ATP-ADO-GSH-Hb and its manufacturing technology have been extensively tested including viral and prion clearance validation studies and various non-clinical pharmacology, toxicology, genotoxicity and efficacy tests. The effects of ATP-ADO-GSH-Hb on appropriate physiological measures in human cell systems, normal animals and disease models have also been determined. The clinical proof-of-concept was carried out in sickle cell anemia patients. The obtained results confirmed that pharmacologic cross-linking of Hb molecules with ATP, adenosine and GSH is highly effective in designing a viable blood substitute for a wide array of therapeutic uses.

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## 17.1 Needs for Blood Substitute

Regardless of previous commercial disappointments, blood substitutes remain the most viable solution to the transfusion medicine problems. There is no doubt that blood transfusions are becoming safer, nevertheless they still carry certain risks. As the infectious risks for HIV and hepatitis have dropped dramatically, newly emerging pathogens are entering the blood supply and reliable screening tests are often not available. The West Nile and Ebola viruses, prion proteins that cause Creutzfeldt-Jakob disease (CJD) and bovine spongiform encephalopathy (BSE), and retroviruses such as xenotropic murine leukemia virus-related virus (XMRV) implicated in chronic fatigue syndrome, pose particular concerns (Klein 2010; Ironside 2006; Stoye et al. 2010). Furthermore, blood transfusions will always carry non-infectious risks ranging from allergic reactions to transfusion related acute lung injury (TRALI), which are potentially life threatening. Today transfusion medicine is on the verge of another problem that may worsen the already existing shortages of stored red blood cells (RBC), particularly in the United States that uses $\sim$14 million units of blood annually. A recent meta-analysis suggests that the transfusion of blood stored longer than 2 weeks carries the risk of death. During storage, RBCs increase their affinity for oxygen and decrease the levels of S-nitrosylated (SNO) hemoglobin (Hb) and adenosine-5′-triphosphate (ATP), which disrupt oxygen delivery and obstruct RBC-regulated vasodilation, as well as generating many oxygen- and lipid-derived toxic factors. These functional and biochemical changes in stored RBC are linked to reduced survival rates of cardiac and trauma patients (Bennett-Guerrero et al. 2007; Lee and Gladwin 2010; Shishehbor et al. 2009; Wang et al. 2012). Another disadvantage of donated blood is the fact that it must be kept refrigerated. Their transfusion requires blood-typing and cross matching that cannot be done at the scene of an accident or on a battlefield. Moreover, the high viscosity and corresponding relatively low flow rate of packed RBCs makes their use highly impractical in the treatment of vascular occlusive episodes (Anderson et al. 1985). Therefore, the simplest solution to these transfusion medicine problems could be an effective blood substitute.

## 17.2 The Quest for an Improved Blood Substitute

Blood substitutes must fulfill strict manufacturing, efficacy and safety requirements. In addition to being pathogen-free, non-toxic, non-immunogenic, non-pyrogenic and having an extended shelf-life, these products should also possess an oxygen carrying capacity sufficient to permit effective tissue oxygenation and adequate retention time. The blood substitutes must be redox inactive and without pressor effects. They should undergo physiologic catabolism. Blood substitutes must be able to rapidly maximize blood flow and tissue perfusion/oxygenation and stimulate erythropoiesis. In the treatment of acute blood loss, an ideal blood substitute should

work as a temporary oxygen bridge until the body is able to restore endogenous RBC mass to maintain adequate tissue oxygenation (Guidance for Industry 2004).

Even though free Hb as potential blood substitute has been sought for decades, to date, the commercialization efforts have been unsuccessful. Almost all clinically tested Hbs caused pressor responses and the redox-active products triggered the inflammatory signaling pathways. A meta-analysis of DCL-Hb (HemAssist® Baxter Healthcare, Round Lake, IL), Hb-Raffimer (Hemolink®, Hemosol, Mississauga, ON, Canada), HBOC-201 (Hemopure®, OPK Biotech/Biopure Corp., Cambridge, MA) and Poly SFH-P, (PolyHeme®, Northfield Laboratories, Inc., Evanston, IL) revealed that these products caused serious side effects in subjects with various co-morbidities. The observed unwanted effects in humans varied from flu-like and gastrointestinal symptoms to heart attack, stroke, and even death (Natanson et al. 2008; Silverman and Weiskopf 2009).

The most accepted scientific explanation of the clinical problems with these products is inevitably their design as only low oxygen affinity carriers with prolonged intravascular persistence without addressing Hb's intrinsic toxicity: a key mediator of the pathological responses seen in human test subjects (Natanson et al. 2008; Silverman and Weiskopf 2009; Riess 2001; Simoni et al. 2009; Simoni 2005; Kluger 2010; Alayash 1999; Buehler and D'Agnillo 2010). In fact, the chemical cross-linkers used for intra- and inter-molecular modification of these products did not possess any heme detoxification ability and heme toxicity always overshadowed the Hb resuscitation benefit (Riess 2001). Thus, inability to control heme toxicity in these products combined with pre-existing conditions that augmented its toxicity in Hb recipients, could be the main reason for these products' ineffectiveness, hindering their commercial development.

These blood substitutes triggered a complex array of pathophysiological reactions as a result of Hb's natural features. Hb is a well-known pressor agent able to react with nitric oxide (NO) and nitrite (NO2) and induce the synthesis of vasoconstrictive factors such as 8-isoprostanes, endothelins, angiotensins and serotonin (Simoni et al. 2009). Hb is a potent oxidant. While in circulation, Hb autoxidizes producing superoxides and reacts with peroxides generating oxygen-, heme- and globin-based radicals (Riess 2001; Simoni et al. 2009; Simoni 2005; Kluger 2010; Alayash 1999; Buehler and D'Agnillo 2010). Acellular Hb by altering the cellular redox state, can activate the nuclear factor kappa B (NF-kappa B) that regulates many inflammatory genes, including cytokines and adhesion molecules (Simoni 2005). Hb and its byproducts may also induce other oxygen and redox regulated transcription factors, hypoxia inducible factor—1 alpha (HIF-1 alpha) and activator protein-1 (AP-1) (Simoni et al. 2009; Simoni 2005; Kluger 2010; Alayash 1999; Buehler and D'Agnillo 2010). The failure of free Hb physiologic catabolism via CD163 scavenger receptor and heme via CD91 receptor, may cause its non-enzymatic degradation and oxidative transformation; negatively affecting a number of vital systems, including renal, cardiovascular, gastrointestinal, neural, immunologic, coagulation, and many others (Simoni et al. 2009; Simoni 2005; Kluger 2010; Alayash 1999; Buehler and D'Agnillo 2010).

All attempts to revive these blood substitute products via protective strategies such as co-administration of nitrovasodilators (i.e., nitroglycerin, nitroprusside, sodium nitrite, etc.) (Moon-Massat et al. 2012; Fonseca et al. 2010) or single antioxidants (Simoni et al. 2009) have been less clinically promising than anticipated, creating incentives for new ideas.

Many academic and industrial centers are already engaged in the development of the next generation of blood substitutes, hoping to prevent the clinical sequelae of free Hb in plasma (Simoni 2012).

## 17.3 ATP-ADO-GSH-Hb: A New Concept in Blood Substitute Design

Texas Tech University Health Sciences Center researchers in order to eliminate Hb's intrinsic toxicity have developed a unique "pharmacologic cross-linking" technique to modify the Hb molecule (Feola et al. 1995; Simoni et al. 2010). This procedure provides Hb with new medicinal properties that eliminate Hb-induced adverse side effects and do not interfere with Hb respiratory function. It addresses problems such as vasoconstriction, oxidation and inflammation *ATP-ADO-GSH-Hb*, is composed of pure bovine Hb, cross-linked intramolecularly with *o*-ATP and intermolecularly with *o*-adenosine, and combined with reduced glutathione (GSH). Such chemical modification procedure besides stabilizing *ATP-ADO-GSH-Hb*'s polymeric structure provides the desired pharmacologic activities of ATP, adenosine and GSH (Fig. 17.1).

The cross-linking reagents aside from possessing the desired pharmacologic activities are classified as "affinity directed" beta-beta cross-linkers. The chemical modification done with these cross-linkers produces a "steering effect" and accelerates the preferential reaction at the beta-beta interface.

In *ATP-ADO-GSH-Hb*, the reaction with ATP stabilizes the Hb tetramer and prevents its dimerization, and the reaction with adenosine allows the creation of Hb polymers with a mass below 500 kDa (Fig. 17.1). ATP besides tetramer stabilization produces the vasodilatory effect through stimulation of the P2Y receptors that are linked to NO and $PGI_2$ production (Ellsworth et al. 1995). This action of *ATP-ADO-GSH-Hb*'s ATP on P2Y is autonomous and not linked with the induction of ATP release from RBCs by Hb-based oxygen carriers with high oxygen affinity and viscosity. Therefore, *ATP-ADO-GSH-Hb*'s ATP is an independent regulator of vascular tone, not affected by P50, pH or shear stress/viscosity (Fig. 17.2a).

The concept of using adenosine was 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.

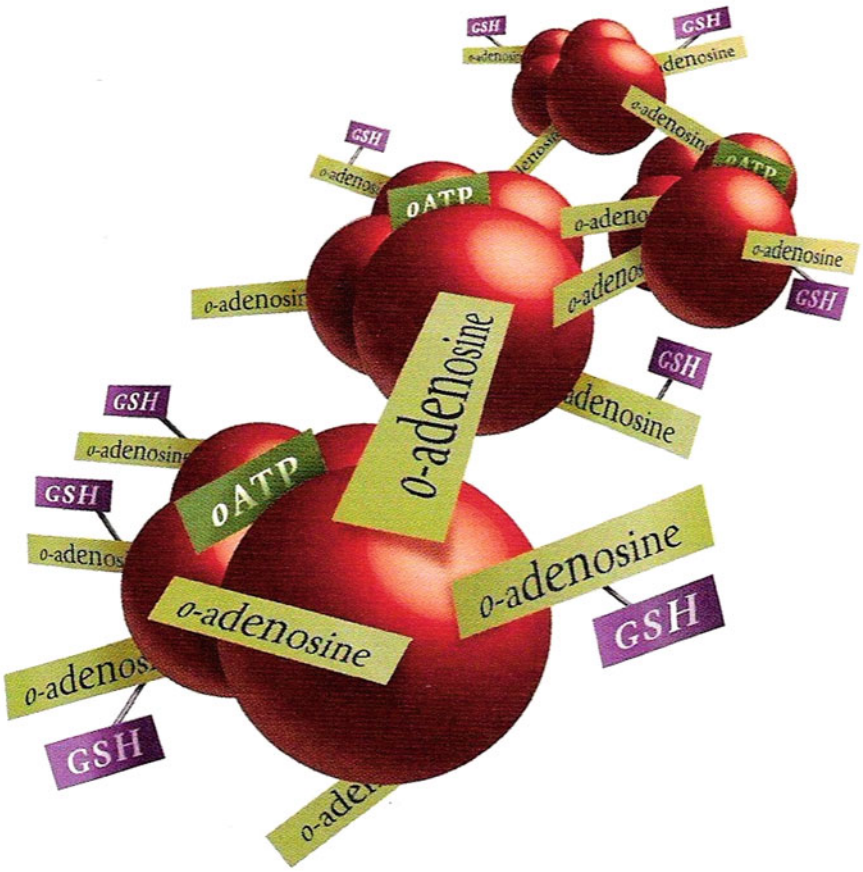


**Fig. 17.1** Graphic Representation of the ATP-ADO-GSH-Hb **Molecule.** ATP-ADO-GSH-Hb, is a pure bovine Hb intramolecularly cross-linked with o-ATP and intermolecularly with o-adenosine, and decorated with reduced glutathione (GSH). This chemical modification procedure stabilizes ATP-ADO-GSH-Hb's polymeric structure and provides the desired pharmacologic activities of ATP, adenosine and GSH

*ATP-ADO-GSH-Hb* reduces the peripheral vascular resistance, an action mediated by the adenosine $A_2$ receptor (Simoni et al. 1996, 1997a, b, 1998, 2000, 2001, 2003), Fig. 17.2b.

The conjugation of Hb with an anionic peptide, GSH, that also has antioxidant properties, was to introduce more electronegative charge onto the surface of the Hb molecule that blocks Hb's transglomerular and transendothelial passage, thus attenuating endothelial pathological responses and nephrotoxic reactions, and makes it less visible to phagocytes (Simoni et al. 1997). At the same time GSH shields heme from the reactive oxygen species (ROS) and NO; therefore lowering the Hb pro-oxidant and vasoconstrictive potential. The *ATP-ADO-GSH-Hb* vasodilatory effect is accelerated by reaction of the endothelial NO with the

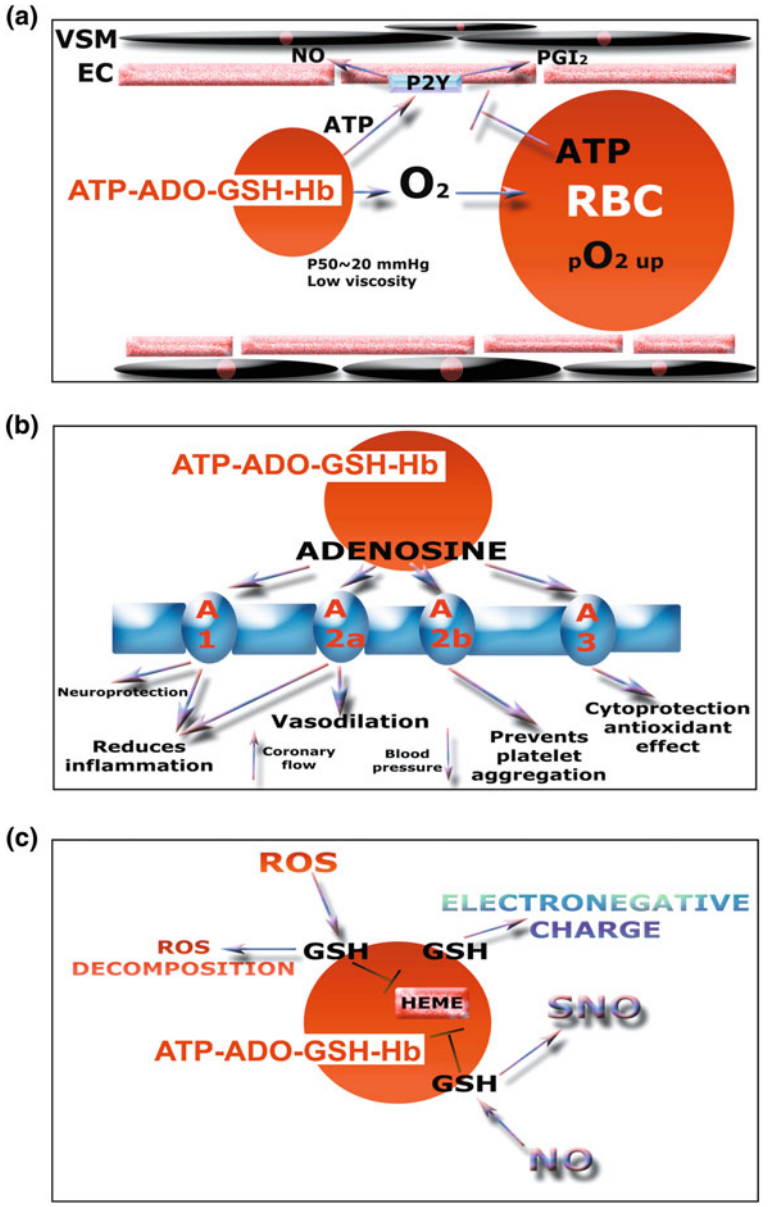


**Fig. 17.2** ATP-ADO-GSH-Hb**'s Physiological and Pharmacological Effects. a Graphic Representation of ATP's Role in** ATP-ADO-GSH-Hb. ATP stabilizes Hb tetramers and produces the vasodilatory effect via stimulation of the P2Y receptors that are linked to NO and $PGI_2$ production. This effect is oxygen and shear independent. **b Graphic Representation of Adenosine's Role in** ATP-ADO-GSH-Hb. Adenosine activates $A_2$ receptors that produce vasodilatation, moderation of inflammatory reactions and prevention of platelet aggregation, $A_3$ receptors that provide cytoprotection, and $A_1$ receptors involved in neuroprotection. **c Graphic Representation of Reduced Glutathione's (GSH) Role in** ATP-ADO-GSH-Hb. GSH introduces more electronegative charges onto the Hb molecule's surface and shields heme from the reactive oxygen species (ROS) and NO; thus lowering Hb's pro-oxidant and vasoconstrictive potential

molecules of GSH, which are chemically attached to the *ATP-ADO-GSH-Hb* surface, producing S-nitroso-GSH. The GSH shield besides preserving NO in its active form also prevented the NO-induced oxidation of heme (Simoni 2005; Simoni et al. 1997, b, c, 1998, 2000, 2012). The alteration of surface charge of *ATP-ADO-GSH-Hb* polymers and tetramers to the same extent is one of the essential features of this novel modification procedure (Fig. 17.2c). The electrophoretic mobility study demonstrated that all Hb polymers have a uniform electronegative surface charge with a pI of 6.1–6.3 (Feola et al. 1995).

*ATP-ADO-GSH-Hb* 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 (Simoni et al. 2001, 2012). *ATP-ADO-GSH-Hb* when reacted with hydrogen peroxide did not oxidatively modify LDLs or cross-link apo B (Simoni et al. 2000). 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 (Simoni et al. 1997, 2000, 2001, 2012).

*ATP-ADO-GSH-Hb* did not change the cellular redox equilibrium nor did it activate apoptotic responses (Simoni et al. 2003). This product was found to eliminate NF-kappa B-induction (Simoni et al. 1997a, b, 1998, 2003). Even in GSH depleted cells; this blood substitute did not activate the inflammatory responses (Simoni 2005; Simoni et al. 2005, 2012). 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 (Simoni 2005; Simoni et al. 1998, 2001). *ATP-ADO-GSH-Hb* also stimulates the adenosine $A_3$ receptor, responsible for cytoprotection (Simoni et al. 2012). It was demonstrated that stimulation of this receptor activates the cellular anti-oxidant enzyme system, thus stabilizing the redox state. In the recent study, using GSH depleted human brain capillary and coronary artery endothelial cells, *ATP-ADO-GSH-Hb* prevented NF-kappa B induction (Simoni et al. 2005).

## 17.4 *ATP-ADO-GSH-Hb* Manufacturing

*ATP-ADO-GSH-Hb* is manufactured at the Texas Tech Blood Substitute Production Facility (Lubbock, TX) using bovine Hb obtained from the blood of a segregated herd of cattle kept at the Texas Tech Animal Blood Donor Unit (New Deal, TX). *ATP-ADO-GSH-Hb* production technology utilizes devices accepted for medical use and USP grade water, crystalloids and buffers, to assure high purity and quality. The *ATP-ADO-GSH-Hb* manufacturing process includes a novel, validated orthogonal technology platform for an effective clearance of prion (CJD/vCJD) and viral pathogens (Simoni et al. 2011a, b). The clearance validation

tests performed in compliance with the Good Laboratory Practice (GLP) standards at BioReliance/Invitrogen Corp. (Rockville, MD), confirmed that *ATP-ADO-GSH-Hb* manufacturing technology is extremely effective in the elimination of prions and non-enveloped and enveloped viruses. While the clearance of 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 (Simoni et al. 2011). The *ATP-ADO-GSH-Hb*'s technology also includes the elimination of endotoxins and steps to ensure product sterility (Feola et al. 1995; Simoni et al. 2010).

*ATP-ADO-GSH-Hb* is formulated as a 6.5 g/dL solution, enriched with electrolytes and mannitol. The final formulation is isotonic and is free of bacterial, viral and prion pathogens and endotoxins (Feola et al. 1995; Simoni et al. 2010, 2012). This product does not contain polymers above 500 kDa and contains less than 5 % of tetramers, and less than 5 % of met-Hb. *ATP-ADO-GSH-Hb* has a uniformed electronegative charge, and P50 of approx. 20 mmHg that maintains a proper oxygen delivery index (Table 17.1).

As a part the Chemistry Manufacturing and Controls Information, ATP-ADO-GSH-Hb was subjected for stability testing at different temperatures (−90, −20, +4, +22, +37 and +42 °C) and time intervals (ranging from days to years). The tests showed that ATP-ADO-GSH-Hb 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 and +42 °C up to 3 days.

ATP-ADO-GSH-Hb is being commercialized by HemoBioTech, Inc (Dallas, TX).

**Table 17.1** Typical Physico-Chemical Characteristics of ATP-ADO-GSH-Hb

| | |
|---|---|
| Oxy-Hb (g/dL) | 6.5 |
| Met-Hb (% of Oxy-Hb) | <5 |
| CO-Hb (% of Oxy-Hb) | <5 |
| pH (U) | 7.8–8.1 |
| Sodium (mmol/L) | 140 |
| Potassium (mmol/L) | 4 |
| Chloride (mmol/L) | 100 |
| Sodium lactate (mmol/L) | 27 |
| Calcium (mmol/L) | 1.3 |
| Mannitol (mg/mL) | 0.8 |
| Colloid oncotic pressure (mmHg) | 20-23 |
| Osmolarity (mOsm/kg) | 300–325 |
| Oxygen affinity P50 (mmHg) | 20–26 |
| Polymeric profile: | <5 % |
| Tetramers (%) | 95 % |
| Polymers < 500,000 daltons (%) | |
| Isoelectric point (pI) | 6.1–6.3 |

## 17.5 Preclinical Performance of ATP-ADO-GSH-Hb

**ATP-ADO-GSH-Hb** has been subjected for an extensive preclinical testing 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 (Simoni et al. 2003). In addition, the effects of **ATP-ADO-GSH-Hb** on appropriate physiological measures in human cell systems, normal animals and disease models have been determined (Simoni et al. 2012).

The pharmacology models used to evaluate **ATP-ADO-GSH-Hb** included: (i) Hemodynamics and tissue oxygen delivery in a hemorrhagic shock model; (ii) Ability to raise cAMP and its reactivity with endothelial NO; (iii) Effects on human blood components; (iv) Anti-inflammatory, anti-oxidant and anti-apoptotic activities toward human endothelial cells; (v) Effects on human brain astrocytes and neurons; (vi) Reactivity with hydrogen peroxide and its ability to participate in the synthesis of vasoconstrictive 8-isoprostanes and endothelins (vii) Transcriptional and translational potential (Table 17.2).

The pharmacokinetics models included: (i) Determination of PK/PD in Coebus monkeys; (ii) Determination of circulatory half-life and renal clearance in Sprague–Dawley rats; (iii) Determination of circulatory half-life and renal and hepatic clearance in New Zealand rabbits (iv) Human monocyte/macrophage clearance; (v) Determination of endothelial clearance and permeability in human coronary artery and brain microvascular endothelial cells (Table 17.3).

The toxicological profile of **ATP-ADO-GSH-Hb** has been determined in: (i) acute toxicity studies in Sprague–Dawley rats and Beagle dogs; (ii) 7-day repeat dose studies in Sprague–Dawley rats and Beagle dogs; (iii) Guinea pig dermal sensitization study; (iv) test for foreign protein in the Guinea pig; (v) standard battery of genotoxicity assays: (a) in vitro mammalian bone marrow cytogenetic test, (b) chromosome aberration in human lymphocytes in vitro, (c) unscheduled DNA synthesis in primary rat hepatocytes.(d) reverse mutation in Salmonella typhimurium, (e) gene mutation in Chinese hamster V79 cells, (f) immunogenicity study (Tables 17.4, 17.5).

Detailed results of these studies have been published (Simoni et al. 2012). The toxicological profile of **ATP-ADO-GSH-Hb**, 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 (Simoni et al. 2012; Tests 1992), (Table 17.4). In acute studies in rats and dogs, **ATP-ADO-GSH-Hb** 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 (Table 17.4). The 7-day repeat dose studies, daily treatment with up to 1,300 mg/kg/day did not result in any significant toxicity (Table 17.4). Multi-dose Guinea pig dermal sensitization studies and tests for

**Table 17.2** Summary of completed pharmacology studies with ATP-ADO-GSH-Hb

| Model | Species | Design/dose | Conclusions |
|---|---|---|---|
| Hemodynamics and tissue oxygen delivery in a hemorrhagic shock model | Rats<br>Male<br>10/group | Rats were injected with 40 % of total blood volume (TBV) of ATP-ADO-GSH-Hb. This randomized study established cardio-vascular responses to ATP-ADO-GSH-Hb and tissue oxygenation | ATP-ADO-GSH-Hb has vasodilatory activity and delivers oxygen to the tissues physiologically |
| Ability to raise cyclic AMP and prostacyclin ($PGI_2$) and its reactivity with the endothelial nitric oxide. Activation of the adenosine and purinergic (P2Y) receptors | Human coronary artery endothelial cells | ATP-ADO-GSH-Hb was tested for the mechanism of its vasodilatory action. Human coronary artery endothelial cells were incubated with 0.4 mM of ATP-ADO-GSH-Hb | ATP-ADO-GSH-Hb significantly increases the production of cyclic AMP, prostacyclin and nitric oxide, acting as agonist for adenosine and P2Y receptors.<br>The nitric oxide decomposition rate in the presence of ATP-ADO-GSH-Hb was significantly lower than with unmodified Hb |
| Effects on human blood components | Human RBC, platelets and LDL | Human RBC, platelets and LDL were incubated with ATP-ADO-GSH-Hb in a concentration corresponding to 40 % TBV | ATP-ADO-GSH-Hb showed no effect on human RBC and LDL. ATP-ADO-GSH-Hb significantly decreased platelet aggregation in response to ADP, arachidonic acid, epinephrine and collagen. The extent of platelet aggregation inhibition was similar to that of adenosine. |

(continued)

**Table 17.2** (continued)

| Model | Species | Design/dose | Conclusions |
|---|---|---|---|
| Anti-inflammatory, anti-oxidant and anti-apoptotic activities toward human endothelial cells | Human coronary artery and brain and lung microvascular endothelial cells | Three human cell culture lines (coronary artery and brain and lung microvascular endothelial cells) were evaluated. 0.4 mM added to each cell culture. The inflammatory responses were evaluated by monitoring the expression of inflammatory cytokines and adhesion molecules. The oxidative reactions were detected by measurement of lipid peroxidation and formation of isoprostanes. Apoptosis was evaluated by phosphatidylserine (PS) externalization and fragmentation of DNA | ATP-ADO-GSH-Hb does not have any pro-inflammatory, pro-oxidative or pro-apoptotic potential in any tested human endothelial cell cultures |
| Effect on human brain astrocytes and neurons | Human brain astrocytes and neurons | ATP-ADO-GSH-Hb was challenged with hydrogen peroxide to determine oxidative potential toward human brain astrocytes and neurons | ATP-ADO-GSH-Hb alone and activated with hydrogen peroxide does not show neurotoxicity |
| Reactivity with hydrogen peroxide and its ability to participate in the synthesis of vasoconstrictive 8-isoprostanes and endothelins | Human endothelial cells | ATP-ADO-GSH-Hb was tested for its reactivity with hydrogen peroxide and its ability to participate in the synthesis of vasoconstrictive 8-isoprostanes and endothelins | The reactivity of ATP-ADO-GSH-Hb with hydrogen peroxide was significantly lower than that of unmodified Hb. ATP-ADO-GSH-Hb does not influence the synthesis of 8-isoprostanes or endothelins |
| Transcriptional, translational and erythropoietic potential | Human microvascular endothelial cells and human astrocytes | ATP-ADO-GSH-Hb was evaluated for its transcriptional and translational potential in vitro using human microvascular endothelial cells and human astrocytes, ATP-ADO-GSH-Hb's was tested for its effect on NF-kappa B, HIF-1 alpha and their respective genes | ATP-ADO-GSH-Hb does not activate nuclear factor NF-kappa B, and therefore does not induce inflammation. ATP-ADO-GSH-Hb stabilizes HIF-1 alpha in normoxic and hypoxic conditions, and therefore accelerates erythropoiesis |

**Table 17.3** Summary of performed pharmacokinetic studies with ATP-ADO-GSH-Hb

| Model | Species/ No. Animals | Design | Conclusions |
|---|---|---|---|
| Determination of PK/ PD | Coebus monkeys Male 6/group | ATP-ADO-GSH-Hb in a volume corresponding to 33 % of TBV was injected I.V. and assessed for structural integrity in plasma at 15 min, 1, 3 and 24 h. The determination of structural integrity of ATP-ADO-GSH-Hb was done with SEC HPLC | In this experimental in vivo condition the circulatory half-life of ATP-ADO-GSH-Hb appears to be approx. 22 h. The structure of ATP-ADO-GSH-Hb was unchanged for up to 3 h. After that ATP-ADO-GSH-Hb's polymers with molecular mass similar to Hb-Hp complex were slowly removed from the circulation. At the 24 h interval, modified tetramers, octamers, decamers and larger molecular structures were still present in the circulation. The spectral analysis at the 24 h interval showed that ATP-ADO-GSH-Hb remained in its reduced, oxy-Hb form |
| Determination of circulatory half-life and renal clearance | Rats Sprague–Dawley Male 10/group | ATP-ADO-GSH-Hb in a volume corresponding to 40 % of TBV was injected I.V. to Sprague–Dawley rats. The level of plasma Hb was used to determine circulatory half-life. The urine Hb concentration was used to determine ATP-ADO-GSH-Hb's renal clearance | At 24 h the concentration of plasma Hb was about half of its initial level; only traces of Hb were found in the plasma after 48 h. There was no detectable Hb in the urine within the experimental period |

(continued)

**Table 17.3** (continued)

| Model | Species/No. Animals | Design | Conclusions |
|---|---|---|---|
| Determination of circulatory half-life and renal and hepatic clearance | Rabbits<br>New Zealand<br>Male<br>10/group | In this model, a volume corresponding to 33 % of TBV was injected I.V. to New Zealand rabbits. The level of plasma Hb was used to determine circulatory half-life. The urine Hb concentration was used to determine ATP-ADO-GSH-Hb's renal clearance. The plasma total bilirubin was used to determine ATP-ADO-GSH-Hb's hepatic clearance | Results indicate that ATP-ADO-GSH-Hb's half-life is approx. 24 h. ATP-ADO-GSH-Hb was not subjected to renal clearance. A significant increase in total bilirubin level suggests its hepatic clearance |
| Human monocyte/ macrophage clearance | Human monocyte/ macrophage | In this in vitro study human monocytes and macrophages were incubated for 24 h with 0.3 mM of ATP-ADO-GSH-Hb | ATP-ADO-GSH-Hb failed to stimulate human monocytes or macrophages |
| Determination of endothelial clearance and permeability | Human coronary artery and brain microvascular endothelial cells | Human coronary artery endothelial cells were incubated for 24 h with 0.4 mM of ATP-ADO-GSH-Hb (clearance study).<br>Human brain microvascular endothelial cells grown to confluence on 0.2 μm membranes were incubated with 0.4 mM of ATP-ADO-GSH-Hb (permeability study) | ATP-ADO-GSH-Hb is not subjected for endocytosis. ATP-ADO-GSH-Hb does not increase vascular permeability |

**Table 17.4** Summary of toxicology studies with ATP-ADO-GSH-Hb

| Design/ species | No. per group/sex | Route/dose groups | Days dosing | Noteworthy findings |
|---|---|---|---|---|
| Acute Sprague–Dawley rat | 11 M and 11 F/group (Three groups) (Two males and two females/group were killed on days 2, 8 and 15 and remaining animals were sacrificed on day 22) | IV (1) 65 mg/ml (20 ml/kg) HemoTech (2) 20 ml/kg isotonic saline (3) Untreated control | 1x on day 1 | ATP-ADO-GSH-Hb was well tolerated in the treated group. Both groups of animals showed piloerection approximately one hour after treatment and reduced activity for approximately two hours. There were not hematological changes between groups throughout the study. Clinical chemistry indicated a slight increase in aspartate transferease on day 2. At necropsy, there were not abnormal observations. No consistent changes were seen on histopathology, although a positive reaction to Perl's stain was seen in the spleen of several animals in both groups, in the liver of one animal treated with ATP-ADO-GSH-Hb |
| Acute beagle dog | 3 M and 3 F/group (3 groups) (one male and one female/group were killed at 24 h and on days 7 and 21) | IV 1) untreated control 2) physiological saline control 3) ATP-ADO-GSH-Hb 65 mg/ml, (19 ml/kg) | 1 | There were no deaths or remarkable clinical signs during the study. Hematology at 24 h showed an insignificant decreased platelet count, which returned to baseline by day 7. Clinical chemistry showed a transient increase in alanine aminotransferase and aspartate aminotransferase at 24 h, which returned to baseline by day 7. 24-hour urinalysis showed no hemoglobinuria. Urinalysis was normal. There were no gross pathological or histological changes noted |

(continued)

**Table 17.4** (continued)

| Design/ species | No. per group/sex | Route/dose groups | Days dosing | Noteworthy findings |
|---|---|---|---|---|
| Repeat dose Sprague–Dawley CD rat | 5 M and 5 F/group (five groups) | IV<br>Three groups<br>ATP-ADO-GSH-Hb: 5, 10, 20 ml/kg/day of 65 mg/mL ATP-ADO-GSH-Hb solution<br>1 group untreated controls<br>1 group physiological saline control: 20 ml/kg/day | Daily x7 | There were no significant clinical observations, changes in hematology, or urinalysis. There were no ophthalmic changes. Clinical chemistry showed a moderate increase in alanine aminotransferase and aspartate aminotransferase on day 5 compared to concurrent controls, however these changes remained within normal levels for this strain of animals. Iron deposition in the spleen was the only abnormality noted during histology |
| Repeat dose Beagle dog | 3 M and 3 F/group (4 groups) | IV<br>3 groups<br>ATP-ADO-GSH-Hb: 5, 10 mL/kg/day of 65 mg/mL ATP-ADO-GSH-Hb solution<br>1 group physiological saline control: 10 ml/kg/day<br>1 group untreated control | Daily x7 | No signs of toxicological significance were observed. ECG revealed no toxicity. No bone marrow abnormalities were noted. Clinical chemistry showed a increase in plasma aspartate activity in some animals at some sampling points, but no consistent effect. Microscopic examination revealed iron deposition in reticuloendothelial liver cells, however there was no degenerative damage |

(continued)

**Table 17.4** (continued)

| Design/ species | No. per group/sex | Route/dose groups | Days dosing | Noteworthy findings |
|---|---|---|---|---|
| Multi-dose Guinea pig | 20 F/group (2 groups) | Intradermal injection<br>**Dose 1** ATP-ADO-GSH-Hb: 100 %, 50 %, 20 %, 10 %, 5 %, 1 % dilution<br>**Dose 2** ATP-ADO-GSH-Hb: undiluted<br>**Dose 3** ATP-ADO-GSH-Hb: undiluted<br>Control: vehicle | 7-day sensitization period followed by 2-day dermal challenge | No contact reactions were observed in the ATP-ADO-GSH-Hb sensitized animals or the negative controls |
| Multi-dose guinea pig<br>Sensitization to foreign protein | 3 F/group (2 groups) | Peritoneal injection<br>Weeks 1 and 2<br>ATP-ADO-GSH-Hb: 0.5 ml IV<br>Week 3 (group 1 only)<br>Week 4 (group 2 only<br>0.2 ml ATP-ADO-GSH-Hb | 3 over 4 weeks | No response was seen in any animal during the induction phase of the study |

**Table 17.5** Summary of genotoxicity studies performed with ATP-ADO-GSH-Hb

| Design/species/strain | No. per group/sex | Route/dose | Days dosing | Noteworthy findings |
|---|---|---|---|---|
| In vitro<br>Reverse mutation in *Salmonella typhimurium*<br>(Ames test) | N/A | ATP-ADO-GSH-Hb: 5,000, 2,500, 1,250, 625, 313 μg/plate<br>Solvent: sterile distilled water<br>+ controls: (1) Sodium azide in water, (2) 9-Aminoacridine in DMSO, (3) 2-Nitrofluorene in DMSO, (4) 2-Aminoanthracene in DMSO | N/A | No toxicity was noted at any concentration in either the presence or absence of S9 activation |
| In vitro chromosome aberration in human lymphocytes | N/A | ATP-ADO-GSH-Hb: 6,500, 3,020, 1,400, 650, 302, 140, 65, 30.2 μg/ml<br>− control: untreated cultures<br>+ controls in sterile distilled water: (1) mitomycin-C, (2) cyclophosphamide | N/A | ATP-ADO-GSH-Hb did not induce increases in chromosomal aberrations compared to controls, either in the presence or absence of S9 activation. |
| In vitro<br>Gene mutation in Chinese Hamster V79 cells | N/A | ATP-ADO-GSH-Hb: 6,500, 3,250, 1,630, 813, 406 ug/ml<br>+ control: (1) ethylmethanesulphonate in ethanol, (2) 7,12-dimethylbenz(a) antracene in DMSO | N/A | ATP-ADO-GSH-Hb did not induce gene mutation in Chinese hamster V79 cells either the presence or absence of S9 activation |
| In vitro<br>unscheduled DNA synthesis (UDS) in primary rat hepatocytes (Autoradiographic Method) | N/A | ATP-ADO-GSH-Hb: 6,500, 2,054, 650, 205, 65.0, 6.50, 2.05 ug/ml<br>Solvent control<br>+ control: 2-acetylamiofluorene in DMSO | 7 | Treatment with ATP-ADO-GSH-Hb did not induce UDS in primary rat hepatocytes under the conditions tested |
| In vivo<br>mouse micronucleus, Swiss, CD-1<br>Femoral bone marrow cells | 1 M and 1 F/ group (5 groups) | IP<br>ATP-ADO-GSH-Hb: 650, 1,300 mg/kg<br>Vehicle: physiological saline<br>+ control: 2.00 mg/kg mitomycin-C | 1 | No significant increases in the frequency of micronucleated cells were observed in the animals treated with ATP-ADO-GSH-Hb |

foreign proteins indicated that **ATP-ADO-GSH-Hb** is not antigenic in this species (Table 17.4). **ATP-ADO-GSH-Hb** did not induce increases in chromosomal aberrations compared to controls, either in the presence or absence of S9 activation (Table 17.5). Also this product did not induce gene mutations in Chinese hamster V79 cells either in the presence or absence of S9 activation (Table 17.5). In vitro unscheduled DNA synthesis (UDS) in primary rat hepatocytes and in vivo mouse micronucleus tests provided further evidence that **ATP-ADO-GSH-Hb** lacks genotoxicity (Simoni et al. 2012; Tests 1992), (Table 17.5).

The results of these pre-clinical studies are favorable, indicating that **ATP-ADO-GSH-Hb** has vasodilatory activity and can reduce vasoconstriction that follows hemorrhage, has erythropoietic activity, and produces no adverse nephrotoxic, neurotoxic, oxidative and inflammatory reactions (Tables 17.2, 17.3, 17.4 and 17.5).

## 17.6 Clinical Performance of ATP-ADO-GSH-Hb

**ATP-ADO-GSH-Hb**'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, Zaire). **ATP-ADO-GSH-Hb** was investigated in nine 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 **ATP-ADO-GSH-Hb** in a volume corresponding to 25 % of TBV no adverse reactions were noted. To the contrary, in children with aplastic crisis, **ATP-ADO-GSH-Hb** 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. **ATP-ADO-GSH-Hb** showed beneficial therapeutic effects in all patients. In fact, **ATP-ADO-GSH-Hb** did not only alleviate the inflammatory reactions in sickle cell anemia patients, but also produced an effective erythropoietic response (Feola et al. 1992).

## 17.7 HemoTech As Multifunctional Therapeutic Agent

The results of preclinical and clinical studies suggest that **ATP-ADO-GSH-Hb** can potentially be used in the treatment of acute and chronic anemias, sickle cell disease, ischemic diseases including myocardial infarction and stroke, and cancer.

**Acute and Chronic Anemias.** A speedy replacement of blood loss with the endogenous RBC seems to be the most attractive feature of **ATP-ADO-GSH-Hb** (Fig. 17.3). The mechanism of erythropoiesis induced by **ATP-ADO-GSH-Hb** was investigated using in vivo and in vitro models, under hypoxic and normoxic

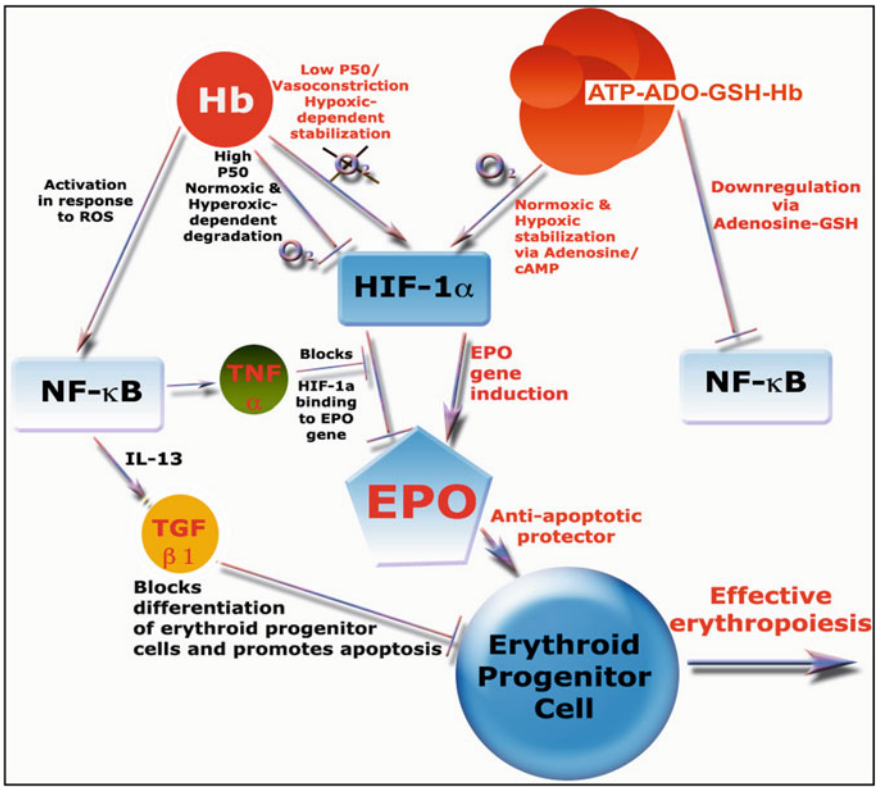


**Fig. 17.3** Graphic representation of the role of ATP-ADO-GSH-Hb and unmodified Hbs (UHb) with different oxygen affinities (P50) in the erythropoietin (EPO) gene induction and the mechanism of erythropoiesis mediated by HIF-1 alpha, NF-kappa B, TNF-alpha, TGF-beta 1, and other factors

conditions. Results indicate that **ATP-ADO-GSH-Hb** as opposed to unmodified Hb, increased the induction of HIF-1 alpha and its binding to the EPO gene and down regulated NF-kappa B, under both oxygen conditions. It was established that adenosine stimulates normoxic induction of HIF-1 alpha and with GSH, inhibits the NF-kappa B pathway that is involved in the suppression of erythroid specific genes, particularly TNF-alpha that blocks HIF-1 alpha binding to the EPO gene and TGF-beta 1 that blocks differentiation and promotes apoptosis of erythroid progenitor cells Simoni et al. (2012b), Fig. 17.3. Therefore, **ATP-ADO-GSH-Hb** can be considered as an Erythropoiesis Stimulating Agent (ESA) useful in the treatment of acute and chronic anemias, particularly those associated with chronic kidney disease and cancer (Simoni et al. 2012).

**Sickle Cell Disease.** This therapeutic indication was tested in humans as part of a proof-of-concept with an excellent outcome and has been published (Feola et al. 1992).

**Ischemic Diseases.** *Myocardial Infarction*: **ATP-ADO-GSH-Hb** was tested on human platelets obtained from percutaneous coronary intervention (PCI) patients and found to decrease platelet aggregability in response to platelet aggregation agonists, particularly collagen, and blocked the release of serotonin (Simoni et al. 2010). Based on this observation and previous studies that proved the ability of **ATP-ADO-GSH-Hb** to optimize oxygen delivery, induce vasodilation, alleviate oxidative reactions and reduce endothelial inflammatory responses (Simoni et al. 2012), **ATP-ADO-GSH-Hb** can be therapeutically useful as a perfusion fluid for PCI procedures. These effects and the prolonged pharmacological action of adenosine (Simoni et al. 2012), the inter-molecular cross-linker that controls vascular tone of the distal coronary arteries, allow **ATP-ADO-GSH-Hb** to also be considered as useful in managing coronary artery disease and myocardial infarction. *Stroke/Traumatic Brain Injury*: The beneficial effects of **ATP-ADO-GSH-Hb** on normal and ischemic human brain capillary endothelial cells, astrocytes and neurons support this therapeutic concept (Simoni et al. 2001, 2003, b, 2012). In fact, **ATP-ADO-GSH-Hb** even supplemented with an excess of hydrogen peroxide, showed to be non-toxic to neurons and astrocytes at any tested concentration (Simoni et al. 2001, 2012). **ATP-ADO-GSH-Hb** also demonstrated a lack of indirect neurotoxicity when exposed to normal and GSH depleted human brain capillary endothelial cells. These observations inspired the concept of its potential use in the treatment of these neurological disorders.

**Cancer.** It is well established that Hb-based oxygen carriers can battle cancer by delivering an excess of oxygen that destabilizes HIF-1 alpha necessary for the induction of angiogenic factors, such as vascular endothelial growth factor (VEGF) (Simoni et al. 2003a, b). Oxygen carriers are also known to be chemo- and radiosensitizing agents (Klein 2002). Therefore theoretically **ATP-ADO-GSH-Hb** can be considered in this application, since its erythropoietic property can treat cancer-associated anemia.

**Other Therapeutic Applications.** Under investigation.

## 17.8 Concluding Remarks

The results of preclinical and clinical studies indicate that **ATP-ADO-GSH-Hb**, a bovine Hb cross-linked intramolecularly with ATP and intermolecularly with adenosine, and conjugated with GSH, provides physiological oxygen delivery and pharmacological properties that counteract the vasoconstrictive, pro-oxidative and pro-inflammatory properties of Hb and provide cytoprotective and erythropoietic effects, all of which are beneficial in combating acute and chronic anemias, sickle cell disease, ischemic diseases including heart attack and stroke, and cancer.

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# Chapter 18
# Albumin-Heme Oxygen Carriers

**Teruyuki Komatsu**

## 18.1 Introduction

Transfusion of red blood cells (RBC) is currently a routine medical treatment because its associated risk of transmission of viral illness has become extremely low. However, (i) cross-matching and compatibility tests must be conducted to avoid a hemolytic reaction in the recipient, and (ii) the storage period of RBC under refrigeration is up to 42 days in Europe and North America, and 21 days in Japan (Hess JR 2006; Otani et al. 2012). These requirements limit the availability of RBC in a disaster or emergency. Several types of hemoglobin (Hb)-based $O_2$ carriers have been developed in the last few decades (Riess 2001; Squires 2002; Winslow 2006; Bettati and Mozzarelli 2011), but unfortunately they do not fulfill all RBC-substitute requirements (Natanson et al. 2008; Kluger 2010; Hess et al. 1993; Sloan et al. 1999).

Human serum albumin (HSA) is the most prominent plasma protein in the circulatory system and has a remarkable capability to bind a broad range of hydrophobic molecules (Peters 2006; Kragh-Hansen 1981, 1990). Typical endogenous ligands for HSA are fatty acids, bilirubin, bile acids, and thyroxine (Curry et al. 1998, 1999; Petitpas et al. 2003; Zunszain et al. 2008). Furthermore, HSA binds many common drugs, such as warfarin, diazepam, ibuprofen (Ghuman et al. 2005). Protoheme IX (**1**) released from metHb is also captured by HSA with a high binding constant ($K = 1.1 \times 10^8\ M^{-1}$) (Adams and Berman 1980). This strong affinity of HSA for heme has stimulated efforts to develop albumin as an artificial hemoprotein which is capable of mimicking the $O_2$ transport of Hb (Monzani et al. 2001). If one could create a new protein hybrid comprising HSA and heme, it would have an important impact not only on RBC alternatives, but also on $O_2$-therapeutic reagents.

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In this chapter, we report on our representative results of albumin-heme $O_2$ carriers, HSA incorporating synthetic heme and recombinant HSA mutant complexing protoheme IX.

## 18.2 HSA Incorporating Synthetic Heme

Our reserach on RBC substitute started from the synthesis of model hemes in attempts to reproduce the $O_2$-binding site of Hb. The first successful example is compound **2** (Fig. 18.1) (Tsuchida et al. 1995). The structure involves (i) a symmetrical tetrakis(phenyl)porphyrin platform, (ii) bulky fences around the coordinated $O_2$, and (iii) an imidazole side-chain covalently bound to the porphyrin. We found that **2** was efficiently incorporated into HSA, and the obtained HSA-**2** hybrid can reversibly bind $O_2$ under physiological conditions (pH 7.3, 37 °C) (Komatsu et al. 1999; Tsuchida et al. 1999).

The solution properties of HSA-**2** ([HSA] = 5 g/dL) are almost identical to those of HSA itself; the specific gravity: 1.013, viscosity: 1.1 cP, and colloid osmotic pressure (COP): 20 Torr. The $P_{50}$ value of HSA-**2** (33 Torr at 37 °C) is similar to that of human RBC (28 Torr) (Table 18.1) (Komatsu et al. 1999; Tsuchida et al. 1999; Imai et al. 1970). Although the $O_2$-equilibrium curve of HSA-**2** does not show cooperativity (Hill coefficient: 1.0), the $O_2$-transporting efficiency between the lungs [$P(O_2)$: ca. 110 Torr] and muscle tissue [$P(O_2)$: ca. 40 Torr] approaches 23 %, which is very close to that for RBC. The $O_2$-association and $O_2$-dissociation rate constants ($k_{on}$, $k_{off}$) were measured by a laser flash photolysis technique (Table 18.1).

Since this achievement, we prepared a series of HSA hybrids incorporating different synthetic heme and evaluated their $O_2$-binding parameters (Komatsu et al. 2001, 2002; Nakagawa et al. 2006, 2008). The $P_{50}$ value can be modulated by tuning the chemical structure of the heme. Compound **3** contains a bulky 1-methylcyclohexanamide group on the porphyrin ring plane (Fig. 18.1), (Komatsu

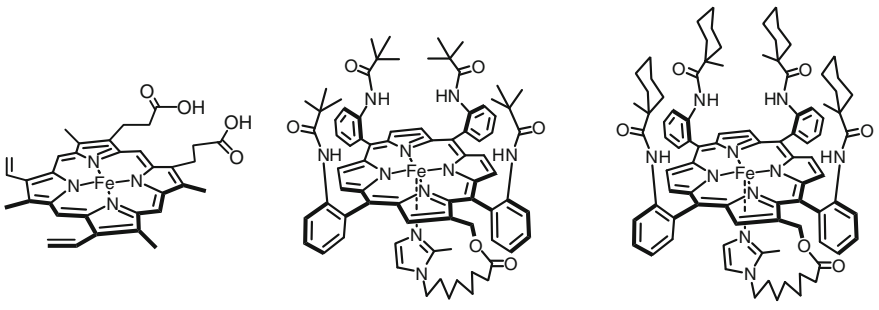


**Fig. 18.1** Structures of natural protoheme IX (**1**) and synthetic hemes (**2**, **3**)

**Table 18.1** $O_2$-Binding parameters of HSA-synthetic heme in phosphate buffer solution (pH 7.3, 25 °C)

| | $10^{-7}k_{on}$ ($M^{-1}s^{-1}$)[a] | $10^{-3}k_{off}$ ($s^{-1}$)[a] | $P_{50}$ (Torr)[b] |
|---|---|---|---|
| HSA-**2** | 34 | 0.75 | 13 (33) |
| HSA-**3** | 46 | 0.98 | 13 (35) |
| $PEG_2$(HSA-**3**) | 12 | 0.17 | 11 (32) |
| $PEG_5$(HSA-**3**) | 12 | 0.17 | 11 (31) |
| RBC[c] | | | 8 (28) |

[a] Presented values are in the fast phase of the kinetics, ref. Komatsu et al. 2002, Huang et al. 2006a. [b] At 37 °C in parentheses. [c] In isotonic buffer (pH 7.4, 20 °C), ref. Imai et al. 1970

et al. 2002). The $O_2$ affinity of HSA-**3** ($P_{50}$: 35 Torr) was the same as that of HSA-**2**, but the stability of the oxygenated state was improved 4.5 times (half-lifetime $\tau_{1/2}$: 9 h).

## 18.3 Poly(ethylene glycol) Conjugated HSA-3

A remaining issue of HSA-**3** is its short circulation persistence in the bloodstream, because the synthetic heme is noncovalently bound to albumin. One possible approach to solve this problem is surface modification with poly(ethyleneglycol) (PEG). It is well known that the decollation of proteins with PEG enhances their plasma half-life, thermostability, and nonimmunogenicity (Veranese and Harris 2002). We inferred that modifying the surface of HSA-**3** with PEG might help to prolong the circulation lifetime and retain its $O_2$-transporting ability in vivo for a long period. Thus, HSA-**3** was modified by maleimide-terminated PEG [$M_w$: $2 \times 10^3$ ($PEG_2$) and $5 \times 10^3$ ($PEG_5$)] (Huang et al. 2006a, b). Mass spectroscopy measurements and quantification of mercapto group of the $PEG_n$-modified HSA-**3** [$PEG_n$(HSA-**3**)] revealed that average six-PEG chains were conjugated on the protein surface. The viscosity and COP of the $PEG_n$(HSA-**3**) solution ([HSA] = 5 g/dL) were controlled by changing the PEG molecular weight. $PEG_2$(HSA-**3**) showed almost the same viscosity and COP as the corresponding value for nonmodified HSA-**3**. In contrast, $PEG_5$(HSA-**3**) demonstrated a higher viscosity (2.3 cP) and more pronounced hyperoncotic properties relative to HSA-**3**.

$PEG_2$(HSA-**3**) binds and releases $O_2$ under physiological conditions (Huang et al. 2006a). The $P_{50}$ value (32 Torr) was not influenced by the presence of the PEG chains (Table 18.1). It is noteworthy that the surface modification by PEG delayed the oxidation of the $O_2$ complex, resulting in a $\tau_{1/2}$ of 12 h, which is almost equal to that of myoglobin (Mb) ($\tau_{1/2}$: 12 h, pH 7 at 35 °C) (Huang et al. 2006a; Sugawara and Shikama 1980).

The persistence of **3** in the bloodstream was measured after administrating $PEG_n$(HSA-**3**) into anesthetized rats (Huang et al. 2006a). The $PEG_n$(HSA-**3**) solution (20 % volume of the circulatory blood) was injected intravenously into rats. The concentration decay of $PEG_n$(HSA-**3**) in the blood showed single

exponentials with a half-lifetime [$\tau_{1/2}$(**3**)] of 13 − 16 h. These values are considerably longer than that observed for a naked HSA-**3**. We can therefore conclude that modifying HSA-**3** with PEG (i) improved its comprehensive $O_2$-transporting ability and (ii) prevented the rapid clearance from the circulatory system.

## 18.4 In vivo $O_2$ Transport by PEG(HSA-3)

We proceeded to evaluate the physiological responses to an exchange transfusion with $PEG_2$(HSA-**3**) into an acute anemia rat model (Huang et al. 2006b). The animals were first placed in a 65 vol % hemodilution with 5 g/dL HSA. They subsequently underwent a 30 vol % blood replacement with the $PEG_2$(HSA-**3**) solution. As negative and positive control groups, a 5 g/dL HSA solution (HSA group) and a washed RBC suspension (RBC group) were infused into similarly operated rats under conditions of hemorrhage. The isovolemic 65 % hemodilution with HSA reduced the Hb concentration, thereby decreasing the supply of $O_2$ to the tissue. As a result, the mean arterial pressure (MAP) and renal cortical $O_2$-partial pressure [$PtO_2$(R)] were decreased (Fig. 18.2 a, e). During hemorrhagic shock by 30 % bleeding, significant decreases in MAP, venous $O_2$-pressure ($PvO_2$), and $PtO_2$(R) were observed by the loss of the circulation blood volume (Fig. 18.2a, c, e). The heart rate (HR) and respiration rate were also decreased (Fig. 18.2b). In contrast, arterial $O_2$-pressure ($PaO_2$) was increased to about 160 % of the basal value (B.V.) (Fig. 18.2d).

The injection of the sample solution increased the blood volume and improved circulatory flow. Lactate was washed out from the tissues and into the circulatory system, which restored the pH to the initial level of 7.43 in all groups (Fig. 18.2f). The administration of HSA did not improve any of these parameters, leading to death within 41 min. On the contrary, the infusion of $PEG_2$(HSA-**3**) or RBC kept all the rats alive until the end of the experiment. After an injection of $PEG_2$(HSA-**3**), the animals showed a marked and rapid recovery in MAP, HR, $PvO_2$, $PaO_2$, and pH, similar to that shown in the RBC group (Fig. 18.2a–d, f). These results demonstrate the $O_2$-transporting capability of the $PEG_2$(HSA-**3**) solution as a resuscitative fluid. It is noteworthy that the HSA-**2** and $PEG_2$(HSA-**2**) $O_2$ carriers did not induce hypertensive action, because of its low permeability through the vascular endothelium in comparison with that of Hb (Huang et al. 2006b; Tsuchida et al. 2003).

## 18.5 Recombinant HSA Mutant Complexing Protoheme IX

Crystallographic studies revealed that natural protoheme IX (**1**) is bound within a narrow D-shaped hydrophobic cavity in subdomain IB of HSA with axial coordination of Tyr-161 and salt-bridges between the porphyrin propionates and a triad

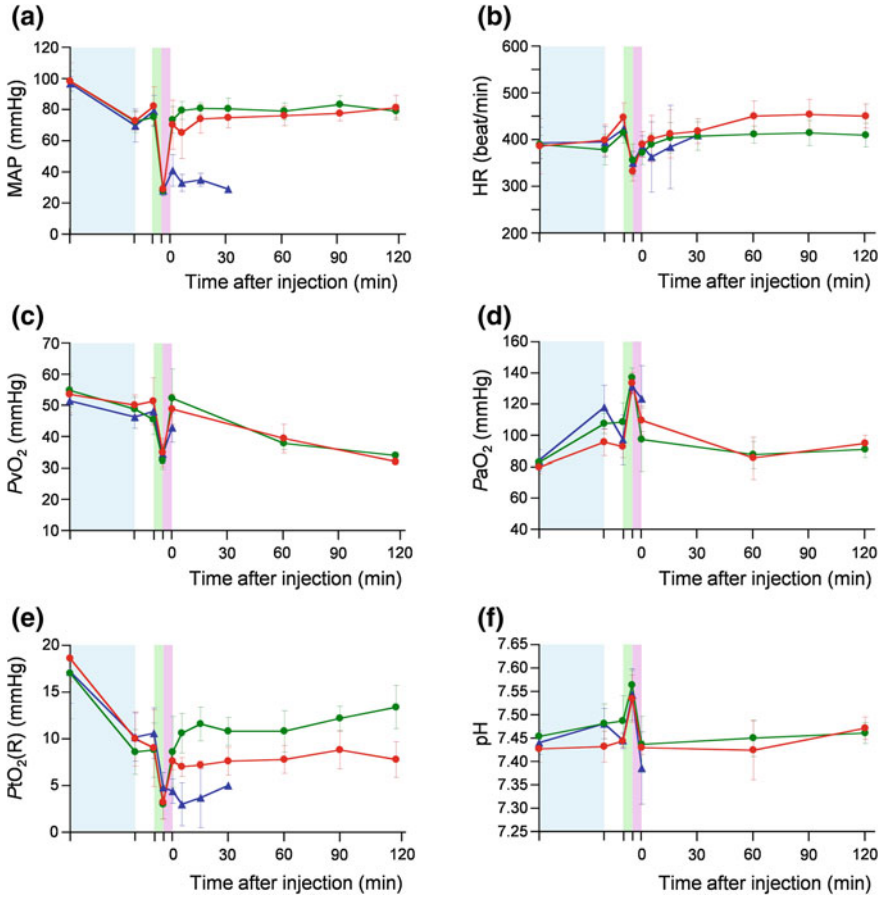


**Fig. 18.2** Effect of $PEG_2$(HSA-**3**) solution on **a** MAP, **b** HR, **c** $PvO_2$, **d** $PaO_2$, **e** $PtO_2$(R), and **f** pH in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SD of five rats [red: $PEG_2$(HSA-**3**) group, green: RBC group, and blue: HSA group].) Light-blue area, light-green area, and pink area, respectively, indicate the period of 65 % hemodilution, 30 % bleeding, and sample infusion

of basic amino acid residues (Arg-114, His-146 and Lys-190) (Fig. 18.3a) (Wardell et al. 2002; Zunszain et al. 2003). In terms of the general hydrophobicity of this α-helical heme pocket, the features of subdomain IB of HSA are similar to the heme binding site of Hb or Mb. However, the reduced ferrous HSA-**1** is rapidly oxidized by $O_2$, even at low temperature (5 °C), because HSA lacks the proximal His which enables the prosthetic **1** group to bind $O_2$. A knowledge of the detailed architecture of the heme binding site of HSA allows us to construct a artificial heme pocket for stable $O_2$ binding. We then used site-directed mutagenesis to introduce an His into subdomain IB of HSA. This would provide axial coordination to the central ferrous ion of **1** and promote $O_2$ binding.

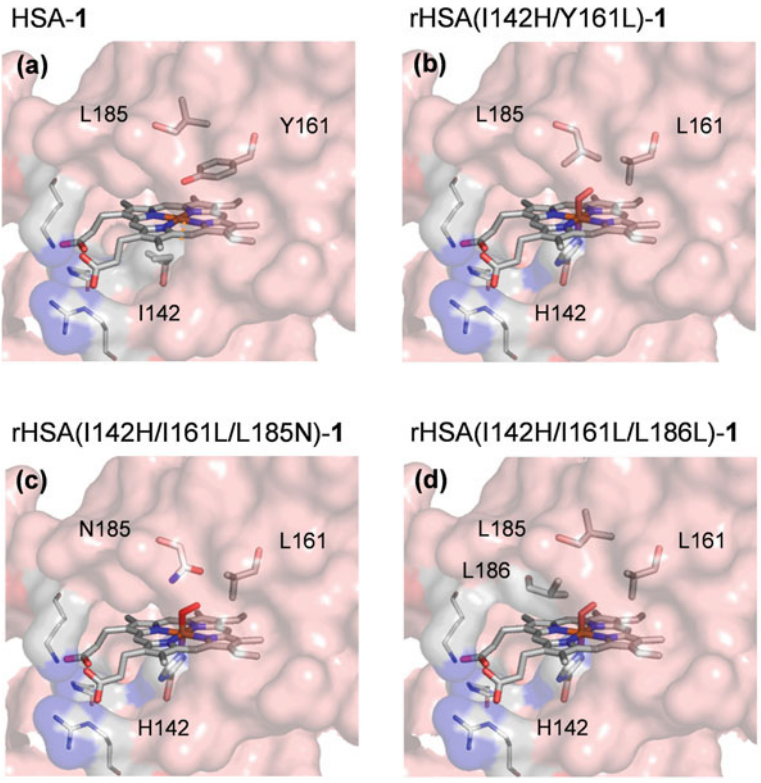


**Fig. 18.3** Structural models of pocket in HSA-**1** and rHSA(mutant)-**1** complexes

Our modeling experiments suggested that the favorable position for the axial imidazole insertion was at Ile-142; the $N_{\varepsilon}$(His) − Fe distance was estimated to be 2.31 Å (compared to 2.18 Å for Mb). We therefore prepared rHSA(I142H/Y161L) mutant and evaluated the $O_2$-binding properties of the resulting heme complex (Fig. 18.3b) (Komatsu et al. 2004; 2005).

The rHSA(I142H/Y161L)-**1** was easily reduced to the ferrous complex under an $N_2$ atmosphere. The single broad Q band ($\lambda_{max}$: 559 nm) in the visible absorption was similar to that of deoxy Hb, indicating the formation of a five-*N*-coordinate high-spin complex (Antonini and Brunori 1971). The heme **1** could be incorporated into the artificial heme pocket with axial His-142 coordination. When the rHSA(I142H/Y161L)-**1** solution was exposed to $O_2$, the UV–vis absorption changed to that of the $O_2$ complex. The $P_{50}$ of rHSA(I142H/Y161L)-**1** was determined to be 18 Torr (Table 18.2). The $O_2$ affinity was 35 − 75-fold lower than those of native Hb and Mb (Gibson 1970; Olson et al. 1971; Rohlfs et al. 1990). This low affinity for $O_2$ was kinetically due to a high $k_{off}$ value.

**Table 18.2** $O_2$-Binding parameters of rHSA-**1** in phosphate buffer solution (pH 7.0, 22 °C)

| | $10^{-6}k_{on}$ ($M^{-1}s^{-1}$) | $10^{-3}k_{off}$ ($s^{-1}$)[a] | $P_{50}$ (Torr)[a] |
|---|---|---|---|
| rHSA(I142H/Y161L)-**1** | 7.5 | 0.22 | 18 |
| rHSA(I142H/Y161L/L185 N)-**1** | 14 | 0.02 | 1 |
| rHSA(I142H/Y161L/R186L)-**1** | 25 | 0.41 | 10 |
| Hb | 33[b] | 0.013[c] | 0.24 |
| Mb[d] | 14 | 0.012 | 0.51 |
| RBC[e] | | | 8 |

[a] Presented values are in the fast phase of the kinetics, ref. Komatsu et al. 44. [b] In 0.1 M phosphate buffer (pH 7.0, 21.5 °C), ref. Gibson 1970. [c] In 10 mM phosphate buffer (pH 7.0, 20 °C), ref. Olson et al. 1971. [d] In 0.1 M phosphate buffer (pH 7.0, 20 °C), ref. Rohlfs et al. 1990. [e] In isotonic buffer (pH 7.4, 20 °C), ref. Imai et al. 1970

## 18.6 Pocket Architecture in rHSA-1

The $O_2$ affinity of rHSA(I142H/Y161L)-**1** is an order of magnitude lower than that of Hb. To develop this artificial hemoprotein as an RBC substitute, its $O_2$ affinity must be enhanced. In Hb and Mb, the distal His-64 stabilizes the coordinated $O_2$ by hydrogen bonding (Phillips and Schoenborn 1981; Shaanan 1983). One approach for increasing the $O_2$ affinity of rHSA(I142H/Y161L)-**1** would be to introduce another polar amino acid at an appropriate position on the distal side of the heme pocket. To test this strategy, we replaced Leu-185 with an Asn, which could act as a proton donor to form a hydrogen bond with the coordinated $O_2$ (Fig. 18.3c) (Komatsu et al. 2007). The rHSA(I142H/Y161L/L185 N)-**1** formed a ferrous five-*N*-coordinated high-spin complex under an $N_2$ atmosphere and produced an $O_2$ complex under an atmosphere of $O_2$.

There is marked difference in a comparison of the $O_2$-binding parameters for rHSA(I1412H/Y161L)-**1** and rHSA(I1412H/Y161L/L185 N)-**1** (Table 18.2). The presence of Asn in place of Leu at position 185 resulted in an 18-fold increase in $O_2$ affinity, because the $k_{off}$ value was decreased by 11 times. The high $O_2$ affinity ($P_{50}$: 1 Torr) for rHSA(I142H/Y161L/L185 N)-**1** is close to those of natural Hb (Gibson 1970; Olson et al. 1971) and Mb (Rohlfs et al. 1990) (Table 18.2).

For administration into the human circulatory system and providing effective $O_2$ transport from lungs to tissues in the body, it would be better for the $O_2$ affinity to be similar to that for human RBC ($P_{50}$: 8 Torr at 25 °C). We recently found that the $P_{50}$ value for rHSA(I142H/Y161L/R186L)-**1** (10 Torr) was almost the same as that of human RBC (Komatsu et al. 2007, 2009).

## 18.7 Conclusions

As described in this chapter, the findings reported herein indicate that the $O_2$-transporting efficacy and initial clinical safety of the HSA-heme were sufficiently positive to permit further advanced preclinical testing. The HSA-heme $O_2$ carriers

might be of great medical importance not only for alternative material of allogeneic RBC transfusion, but also for $O_2$-providing therapeutic fluid in various clinical situations. The HSA is now produced on an industrial scale (Kobayashi 2006), which would permit its use in albumin-based $O_2$ carriers in practical applications.

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# Chapter 19
# Recombinant Human Hb-SOD Fusion Proteins

**Marie Grey, Khuanpiroon Ratanasopa and Leif Bülow**

## 19.1 Togetherness Between Proteins

During the last decade, the protein composition of red blood cells (RBCs) has been extensively studied by several research groups. Such studies have involved both the cytosolic protein fraction and the membranes. Most of the cytosolic RBC proteome has today been analyzed in detail. Since mitochondria are lacking, RBCs need to produce ATP by glycolysis, which is followed by lactate fermentation of the formed pyruvate (Walpurgis et al. 2012). Enzymes participating in these metabolic pathways such as glyceraldehyde-3-phosphate dehydrogenase or lactate dehydrogenases are therefore key proteins in the RBC cytoplasm. In addition, RBCs have to cope with high levels of oxidative stress due to their transport of oxygen (Cimen 2008; Hattangadi and Lodish 2007). To ensure protection against reactive oxygen species, different cytosolic proteins such as peroxiredoxin 2, superoxide dismutase 1 and catalase, which all have successfully been identified using mass spectroscopic techniques, need to be functional. RBCs also harbor a network of skeletal proteins that provides high flexibility and elasticity to the surrounding membrane (Mohandas and Gallagher 2008).

Such proteomic studies of RBCs, as well as of other cells, have clearly demonstrated not only the presence of specific proteins, but they have also shown that these proteins are structurally organized. The concept and view of the RBC as a simple bag of hemoglobins and other necessary proteins need to be abandoned for a more complex picture where protein organization is essential for many metabolic processes involving different pathways and cellular protective systems. A number of proteins thus often operate together in multi-step reactions or cascade processes. In these metabolic pathways, the product of one enzyme is the substrate of the

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other. Alternatively, a toxic compound can be effectively removed by such spatial organization before it reaches the surrounding environment. Activity is commonly regulated by protein organization at the molecular level. Not only are cooperating proteins often localized in the same compartment, they can also be associated in macromolecular complexes, which can be formed by covalent bonds or be more loosely and transiently generated (Candau et al. 1991; Negrutskii and Deutscher 1992; Srivastava and Bernard 1986). The organization of proteins in these assemblies has a major advantage which often is referred to as metabolic channeling where the active sites of the proteins/enzymes are brought in close proximity to each other. In this way the intermediate compound is transferred from one catalytic site to the other without or at least with less diffusion into the bulk phase of the cell. Furthermore, the complexes maintain high local concentrations of the metabolites, which is crucial for unstable and harmful compounds. As the concentration in the rest of the cell is low, toxic intermediates are thereby rendered much less dangerous when being concentrated to a specific microenvironment close to a given protein surface.

Multiprotein aggregates able to catalyze several separate catalytic reactions have been characterized as either multienzyme complexes or multifunctional enzymes. A multifunctional protein is composed of a single polypeptide chain carrying two or more active sites. A multienzyme complex also harbors several active sites, however, each on distinct polypeptide chains. Expressions like protein machines, enzyme clusters, supramolecular complexes, aggregates, and metabolons, all refer to multienzymes. Recommendations for the nomenclature of these multienzymes have been established and generally accepted (Nomenclature for Multienzymes 1989).

## 19.2 Hb Reactivity

Hb binds $O_2$ reversibly to a highly reactive heme group. The problem with this arrangement is, however, the redox active metal ($Fe^{2+}$) in the heme group which can give rise to undesired redox reactions. When these reactions involve hydrogen peroxide ($H_2O_2$), high oxidation states of the iron and free radicals are formed, see Fig. 19.1. Both the ferryl iron ($Fe^{4+} = O^{2-}$) and the globin bound free radical are able to participate in oxidative reactions which result in oxidative damage to tissues such as lipid peroxidation and protein modification (Carlsen et al. 2005; Olsson et al. 2012; Reeder 2010; Vollard et al. 2005).

Superoxide radicals and $H_2O_2$, which are harmful reactive oxygen species (ROS), are produced by autoxidation of Hb. Another source of superoxide anions is re-oxygenation of a tissue previously deprived of oxygen, a phenomenon known as reperfusion injury (Alayash 2004). Furthermore, $H_2O_2$ is produced from platelets, neutrophils and macrophages resulting in plasma levels around 0.25 μM (Alayash 1999; Jia et al. 2007). The $H_2O_2$ may initiate a redox cycle between ferric and ferryl Hb eventually leading to heme degradation and free iron. The free iron

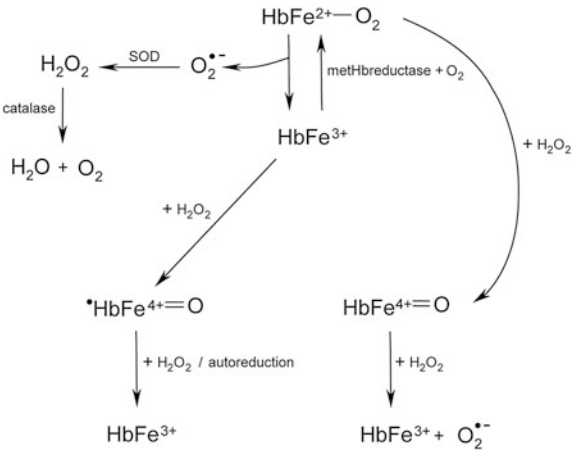


**Fig. 19.1** Autoxidation of Hb as well as reacting with $H_2O_2$ can result in high oxidation states of Hb. In the body, enzymes such as superoxide dismutase (SOD) and catalase detoxify superoxide radicals and $H_2O_2$. Ferric Hb ($HbFe^{3+}$) is reduced back to ferrous ($HbFe^{2+}$) using metHb reductase

can then initiate Fenton chemistry, resulting in hydroxyl radical formation. This radical is extremely reactive; its reactivity is similar to that of ferryl Hb and the globin bound radical, and has been implicated in e.g. DNA and cellular damage (Baldwin 2004; Everse and Hsia 1997; Vollard et al. 2005).

## 19.3 Superoxide Dismutase

Superoxide dismutases (SOD) belong to our most important defence systems against oxidative damage as they catalyze the dismutation of superoxide radicals to $H_2O_2$ according to equation 19.1. Catalase then converts the $H_2O_2$ formed to water and oxygen.

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{19.1}$$

Humans possess three different types of SOD; the dimeric copper/zinc-SOD present in the cytosol, the tetrameric manganese-SOD located in the mitochondria and the tetrameric glycosylated SOD present extracellularly. Under normal circumstances the SODs can effectively cope with the superoxide anions produced. However, under pathological conditions too much superoxide anions may be produced overwhelming the natural defence systems. SOD dysfunction or the superoxide anion radical itself have been implicated in several conditions such as ischemia (shortage of blood supply), cancer and diabetes (McCord and Edeas 2005). Therapeutic use of SOD has therefore been suggested and also clinically tested with some success (Gao et al. 2003).

As indicated earlier, in the RBC, Hb is surrounded by antioxidant enzymes including catalase, superoxide dismutase (SOD) and metHb reductase in addition to antioxidants such as ascorbate and glutathione. These protect the surrounding

tissues as well as the Hb itself from oxidation (Baldwin 2004). Nevertheless, administration of Hb as a blood substitute may overwhelm these protective systems. The catalase present intracellularly may be sufficient to remove $H_2O_2$ by conversion to water, however, there is not sufficient SOD to eliminate the superoxide radicals (Alagic et al. 2005). Fortunately, there are several approaches that can be used to detoxify ROS and the cytotoxic ferryl Hb. One method is based on the protein conjugates composed of SOD and catalase together with the Hb, an option explored by several groups (Alagic et al. 2005; Chang 2008; Grey et al. 2009; Isarankura-Na-Ayudhya et al. 2010; Tarasov et al. 2007). Moreover, addition of certain reductants (iron chelators) has been shown to reduce the ferryl state to the less toxic ferric (met) state (Reeder et al. 2008).

## 19.4 SOD-Haemoglobin

Proximity or "togetherness" is essential when examining proteins having a cooperative action. Such closeness between SOD and Hb can be generated by three different approaches:

1. Co-immobilization of the proteins to a carrier material.
2. Chemical cross-linking of the proteins.
3. Preparation of artificial bi- or polyfunctional proteins by gene fusion.

Particularly chemical cross-linking has been explored for generating proximity between SOD and Hb. Hb has previously been chemically conjugated to SOD alone or SOD combined with catalase—Hb-SOD and polyHb-SOD-CAT (Alagic et al. 2005; D'Agnillo and Chang 1998; Tarasov et al. 2007). The latter Hb conjugate has been shown to have anti-oxidative properties and offer protection against reperfusion injury (Powanda and Chang 2002). However, chemical conjugation has several drawbacks: the cross-links are introduced at different amino acid residues and usually both intra-tetramer as well as inter-tetramer bonds are formed with multiple side chains involved. In addition, reactive groups are introduced, leading to the need of including blocking steps in the process (Alagic et al. 2005; Ronda et al. 2008).

To avoid these problems a gene fusion approach has been explored (Bulow and Mosbach 1991; Grey et al. 2009). The structural genes of the proteins of interest are fused in-frame generating a recombinant polypeptide chain carrying both active sites when the novel gene is expressed in suitable host cells. Fusions can be made either to the amino- or carboxy-terminal ends of the proteins depending on known restrictions in the tertiary or quaternary structure of the protein. In particular, the use of polymerase chain reaction (PCR) technology has greatly facilitated the construction of these artificial bifunctional proteins. Alternatively, suitable restriction enzyme sites necessary for further genetic manipulations can be generated at the 5′- or the 3′- regions of the structural genes by site-directed mutagenesis. Moreover, by using chemically synthesized DNA fragments in the

cloning procedure, special properties in the linker region between the enzymes can be introduced by the selection of a certain oligonucleotide sequence. The chimeric gene prepared is then inserted into a proper expression vector and transformed into a suitable host cell.

In many instances, the use of this genetic approach is advantageous over the immobilized and cross-linked enzyme systems. Large amounts of homogeneous bifunctional protein can be produced in a cost effective process, whereas the degree of cross-linking and homogeneity may vary between different preparations of chemically prepared protein conjugates. Additionally, much of the enzyme activity is often lost in the immobilization or cross-linking procedure that is normally not the case for gene fusion. Most often at least 50 % of the wild-type enzyme activity is retained if the entire primary structure of the native enzyme is maintained in the hybrid enzyme prepared (Bulow et al. 1985; Lindbladh et al. 1991; Lindbladh et al. 1994; Ljungcrantz et al. 1989). However, the ratio between the protein activities is generally fixed in a genetically prepared system while it is easy to change it using the other two methods. However, the gene fusion technique may be modified and needs not to be limited to a simple 1:1 ratio but can easily be extended to other ratios when the fusion protein is designed. For instance, the activity of the detoxifying enzyme (e.g. SOD) can be increased by fusing the corresponding gene directly after each other in a tandem fashion (Kuchinke and Muller-Hill 1985; Lindbladh et al. 1987) or by placing the enzyme in both the N- and C-terminal ends of the hemoglobin protein.

By using this gene fusion method, the link between the SOD and Hb is always present between the same amino residues, thereby avoiding heterogeneity. The construct has been prepared by fusing the human manganese SOD and Hb α-chain genes (Fig. 19.2). Manganese SOD has been chosen due to its longer half-life in serum and lower product inhibition by hydrogen peroxide (Borgstahl et al. 1992;

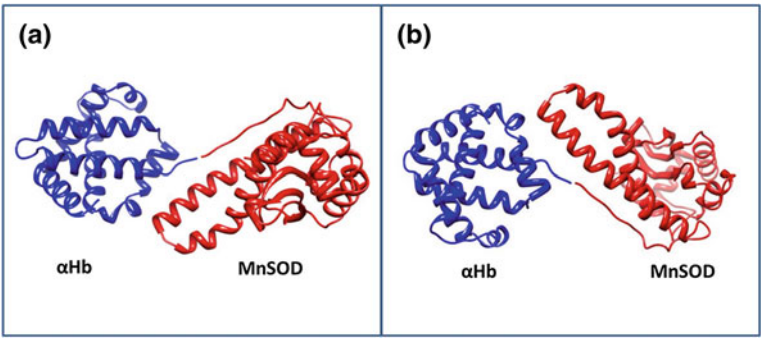


**Fig. 19.2** A modelled structure of the Hb-SOD fusion protein obtained using protein docking at the HADDOCK webserver (de Vries et al. 2010). The starting structure for docking is 2DN1 and 1AZP for HbA and human MnSOD, respectively. The UCSF Chimera program was used to create the final figure (Pettersen et al. 2004). An alanine residue is located in between the two native protein structures

Gorecki et al. 1991). The SOD-Hb has been designed with only one alanine residue in the linker in order to promote proximity, increase stability as well as to reduce potential proteolytic degradation during production. In reactions with ROS, proximity between SOD and Hb gives favourable protection for the Hb (Grey et al. 2009). Similarly, when using the chemical cross-linking approach, it has been shown that shorter chemical cross-links may provide enhanced stability to Hb (Bobofchak et al. 2008).

## 19.5 Characterisation of SOD-Haemoglobin Fusion Proteins

In the oxidative reactions shown in Fig. 19.1, Hb is both perpetrator and victim of its own reactivity. ROS both damage the Hb and surrounding tissues, in addition Hb is rendered useless as $O_2$ carrier. Both superoxide radicals and $H_2O_2$ increase the rate of ferric Hb formation (D'Agnillo and Chang 1998) and eventually lead to heme degradation. The SOD-Hb fusion protein thus has the potential to protect not only tissues but also Hb itself.

In our first construct, we have made a fusion between the C-terminus of the α-chain of hemoglobin and the N-terminus of the SOD. This chimeric construct was coexpressed with Hb β-chains in *E. coli* (Fig. 19.3). SOD-Hb retained normal Hb functionality showing typical absorption spectrum characteristics of human Hb in both oxy- and CO-form. Additionally, SOD-Hb autoxidized considerably slower than Hb as the rate constant was decreased to almost half; 0.10 $h^{-1}$ compared to 0.18 $h^{-1}$ for native HbA. The SOD-Hb also proved to have higher thermal stability than Hb. However, the fusion protein shows an interesting subunit arrangement. SOD is a tetrameric protein which in turn affects the oligomeric nature of the fusion protein. Large polymeric protein aggregates are obtained together with

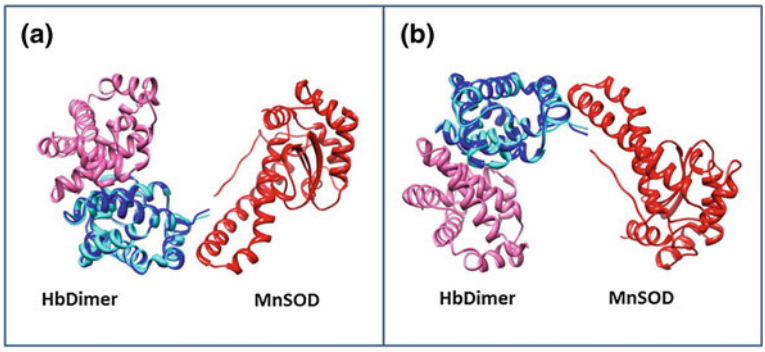


**Fig. 19.3** Modelled structure of the fusion protein SOD- αHb expressed together with the native β-subunits of Hb

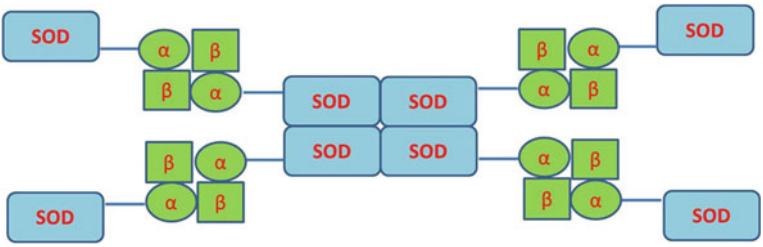


**Fig. 19.4** High molecular weight structure of SOD-Hb determined using gel filtration on a Superdex 200 preparative grade column. Note that the Hb $\beta$-chain is associated with the SOD-$\alpha$Hb fusion in all conformations

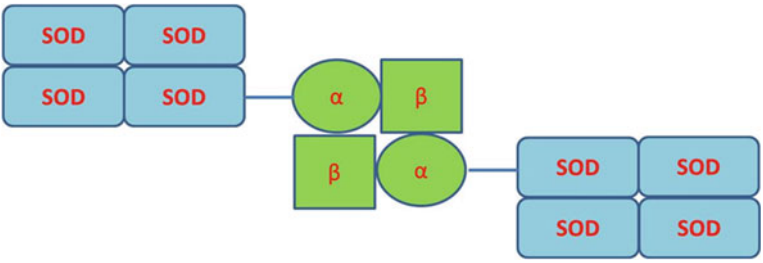


**Fig. 19.5** Coexpression together with native SOD subunits results in a homogeneous SOD-Hb fusion protein

fractions containing individual SOD subunits (Fig. 19.4). This first generation SOD-Hb fusion protein retains approximately half (52 %) of the native SOD activity. As native SOD is functional as a tetramer, the dimer and monomer fractions of SOD-Hb exhibit much lower activity (Borgstahl et al. 1992, Borgstahl et al. 1996). Even though full SOD activity apparently is not necessary, gel filtration could be used to remove inactive fractions in addition to produce a more homogeneous product. The aggregation pattern is thus complex and in order to generate a homogeneous fusion protein, we also added native SOD subunits in our *E. coli* expression system. These separate free subunits can interact with our chimeric protein forming a fusion protein with an SOD activity close to the native one (Fig. 19.5).

## 19.6 Protective Properties of SOD-Hemoglobin

*In vitro* tests have proved that the SOD moiety of the fusion protein is able to protect the Hb molecule. When challenged with a xanthine/xanthine oxidase superoxide generating system SOD-Hb is seven-fold more effective in reducing the amount of ferryl Hb formed compared with the native form of Hb. In order to

further dissect the importance of proximity between SOD and Hb in such protection, we setup an identical system of native Hb and SOD with matched activities. The protection was less, only 50 % of the ferryl reducing activity was obtained compared to having the two proteins close together.

Similarly, the $H_2O_2$ produced during autoxidation can result in heme degradation when reacting with Hb. Heme degradation can be followed via the fluorescent degradation products formed. When challenged with $H_2O_2$ *in vitro*, SOD-Hb was shown to exhibit considerably lower heme degradation. As expected higher $H_2O_2$ concentrations gave more heme degradation, although always at least 50–60 % lower for SOD-Hb compared to Hb alone.

The physical stability of a fusion protein is always a concern as the stability may be influenced by e.g. folding problems or hindrances in subunit arrangements. Thermal stability was therefore investigated using differential scanning calorimetry (DSC). Denaturation of native Hb begins with the dissociation of the tetramer into monomers, ending with a certain degree of aggregation (Michnik et al. 2005). SOD-Hb shows a broader, less well-defined peak than Hb, indicative of a complex denaturation path with contributions from several thermal processes. Interestingly, SOD-Hb has greater heat stability than Hb as apparent $T_m$ values were found to be 55.1 °C for SOD-Hb, considerably higher than that of Hb (48.7 °C).

## 19.7 Conclusions

The fusion protein SOD-Hb displays several advantages compared to the corresponding native proteins or a chemically cross-linked system, that makes it interesting for practical applications. These advantages include proximity effects, improved thermostability due to the fusion and benefits in the purification and production of the proteins since only one protein needs to be handled instead of two. The fusion protein can furthermore be seen as a ready-made protective system suitable for entrapment in e.g. vesicles to generate a new generation of blood substitutes.

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# Chapter 20
# In-Vitro Production of Functional RBCs from Hematopoietic Stem Cells

**Eun Jung Baek and Hyun Ok Kim**

## 20.1 Introduction

A great deal of research has focused on methods to produce RBCs industrially in order to reduce reliance on human donations. Among the possible candidates for artificial blood, the most perfect substitute would be actual human RBCs derived from erythroid progenitor cells grown in vitro replicating production *in vivo*. Even though this idea has been around for several dozen years, it has only become feasible since the 2000s, when the stem cells were brought up in conversation. In the last decade, substantial advances have been made toward the goal of creating artificial blood in vitro. Research has progressed to large-scale production, and created the establishment of various stem cell sources for RBC production, automated blood manufacturing bioreactors, and competitive pricing of RBC components.

The first attempted in vitro mass production of RBCs was demonstrated in Luc Douay's work in 2005 (Giarratana et al. 2005). This showed that a large quantity of RBCs could be made from adult and fetal hematopoietic stem cell (HSC) sources via purely differentiated erythroblasts. Other researchers later developed culture methods that did not make use of feeder cells, which have been commonly used to support erythropoiesis processes that occur in bone marrow (BM) macrophages and the extracellular matrix (Miharada et al. 2006). It was found that all serum, plasma, and stromal cells could be removed from the culture conditions (Kim and Baek 2012). Recently, human embryonic stem cells (ESCs) and induced

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pluripotent stem cells (iPSCs) were used for RBC production to provide an unlimited source of stem cells (Ma et al. 2008; Lapillonne et al. 2010). Currently, several teams are developing automated culture systems for large-scale blood manufacturing (Housler et al. 2012). The first human transfusion of in vitro-generated RBCs derived from G-CSF mobilized autologous peripheral blood HSCs was attempted in 2011 (Giarratana et al. 2011). The amount of RBCs transfused was equivalent to 2 mL of blood.

Even though there are still many hurdles in replacing the current blood donation system, the in vitro generation of RBCs from HSCs is a promising solution to the shortage of safe donated blood for transfusion. In this chapter, we will briefly introduce erythropoiesis and discuss the history and the future of RBC manufacturing.

## 20.2 In Vivo Production of RBCs in BM

The site of hematopoiesis change multiple times during embryo development. For the 2 months from embryo formation to implantation in the uterus, hematopoiesis occurs in the yolk sac (Manwani 2008). After this initial stage, hematopoiesis occurs primarily in the liver and the spleen until the fetus is 7 months old. From then until 2 weeks after birth, hematopoiesis continues to occur in the liver and the spleen and also begins to occur in the BM. The location of hematopoiesis then gradually shifts to the BM. Then, by 6 months after birth, the BM becomes the main location of blood cell production, which includes RBCs, platelets, and granulocytes.

Other cells such as stromal cells, trabeculae bone, and vessels fill supportive roles for hematopoiesis in the BM. In addition, other adhesive extracellular matrices, such as fibronectin and laminin, influence erythroid cell maturation (Housler et al. 2012).

Mature erythroid cells in the BM are formed starting with HSCs. These give rise to burst-forming unit erythroid (BFU-E), which give rise to colony-forming unit erythroid (CFU-E), which in turn give rise to proerythroblasts. From the CFU-E stage, erythropoietin is the most important cytokine regulating erythropoiesis. Upon the stimulus of erythropoietin, a series of changes occurs including hemoglobin concentration, cell size minimization, and chromatin condensation, as cells differentiate to basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts. The last erythroblasts enucleate their nucleus and become reticulocytes, which still have micro-organelles such as ribosome and mitochondria. In this phase, reticulocytes leave the BM and enter the peripheral blood circulation, then become donut-shaped RBCs. From HSCs to mature RBCs, one stem cell could divide about 20 times, finally making more than $10^6$ mature cells. Under normal conditions, more than $2 \times 10^{11}$ RBCs are generated in a human adult every day (Gordon et al. 2002), and this number can be as high as eight times more in an acute anemic state (Goodnough et al. 2000). The process to develop from HSCs to RBCs takes about 7 days.

## 20.3 In Vitro Production of RBCs from Cord Blood- or Adult-Hematopoietic Stem Cells

To produce human RBCs in vitro, the most common starting material is HSCs, as in the BM. They can be isolated using anti-CD34 antibodies from umbilical cord blood, bone marrow aspirates, or the G-CSF mobilized peripheral blood. However, umbilical cord blood is the least invasive and most easily acquired source, and it has frequently been studied for in vitro erythropoiesis. The complicated structures and composition of the BM are mimicked in the in vitro culture environment. For example, researchers usually co-culture hematopoietic and erythroid cells on plate-attached stromal cells, such as macrophages and other stromal cells.

In 2004, Giarratana et al. showed the large-scale in vitro production of RBCs (Giarratana et al. 2005). Following this pioneering work, there have been a number of related studies, and the protocol in general comprises three steps: (1) CD34+ cells are isolated and stimulated to proliferate and differentiate to the erythroid lineage in the presence of three to five cytokines (e.g., stem cell factor, erythropoietin, interleukin-3) for 1 week, (2) immature erythroid cells are expanded and matured by erythropoietin in iron-containing media for another week, (3) terminally matured erythroid cells are co-cultured on stromal cells and enucleated (Figure 20.1).

Giarratana et al. (2005) used immortalized murine cell lines to mimic macrophage-erythroblasts interactions in BM. However, the use of xenogeneic cell sources is not feasible for human use due to the risk of inter-species contamination. Moreover, it is very difficult to prepare the huge amount of stromal cells needed and to accommodate the space needed for co-culture.

In 2006, Miharada et al. (2006) succeeded in RBC production in the absence of feeder cells. However, they used human serum, which is impractical for large-scale production of RBCs. A few years later, (Kim and Baek 2012) developed feeder-free and serum/plasma-free RBC production methods that are applicable to conventional bioreactor generation systems and that are safe and cost efficient. However, the cell-to-cell signals and the secreted paracrine stimuli from stromal cells were insufficient, and the technique did not achieve high enucleation rates.

Enucleation is the process by which mature orthochromatic erythroblasts extrude their nuclei, and is currently regarded as an asymmetric cell division. However, many aspects of this process and its related mechanisms remain unclear. In experimental conditions, enucleation is not efficient, likely due to accumulated cell stress and the lack of proper signals, supporting cells, and nutrients. In circulation, the extruded nuclei, which are fragile and inflexible, are removed by spleen macrophages. The free nuclei or nucleated RBCs which are not removed from the final cell products might pose leukemogenic or dysplastic risks. In addition, the condition of the recipient should be considered in terms of whether the nucleated erythroblasts and extruded nuclei in peripheral blood could be safely removed by the spleen without side effects. Therefore, the final RBC product must be thoroughly evaluated for remnant nuclei and overall safety. Most importantly, a method to enucleate erythroblasts should be developed.

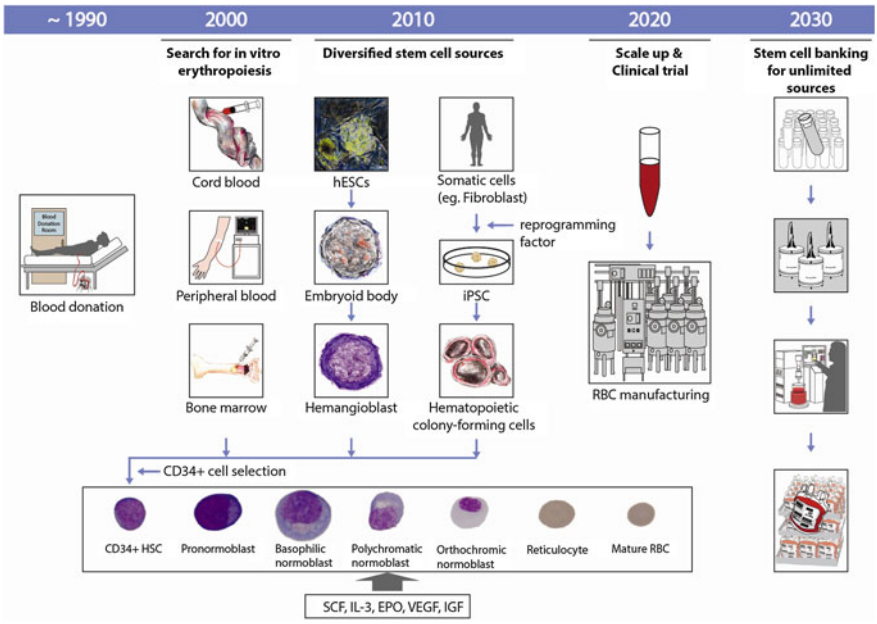


**Fig. 20.1** A schematic diagram of the history and the future of RBC manufacturing

## 20.4 RBC Production from Human ESC or iPSC

To provide a large quantity of RBCs, plentiful stem-cell sources would need to be established. However, methods to maintain the self-renewal capacity and regeneration of HSCs derived from BM, cord blood, or mobilized peripheral blood have not been developed for use *in vitro*. Therefore, researchers have attempted to produce RBCs from ESCs and iPSCs.

In 1998, human ESC lines have been established, and there has been some success in maintaining their 'stemness' (Thomson et al. 1998). 10 years later, several groups (Lu et al. 2008, Ma et al. 2008) showed that erythroblasts could be generated from ESCs, but the RBC yield was very low, with just a few RBCs from each ESC.

Since there is no established hESC line with the O Rh-negative blood type, and since a variety of cell materials with specific blood types are needed, iPSCs have emerged as the most promising cell source. iPSCs are 'ESC-like' cells which can self-renew indefinitely in vitro and have the ability to differentiate to the three germinal layers. To establish iPSC sources for universal donor blood, donors would need to be O Rh-negative and be without any other minor immunogenic antigens such as Rh subtypes Lewis, Kell, Duffy, and Kidd, which are likely to evoke immune reactions.

Potentially, iPSCs could be cultured from patients who require a very specific blood type due to a genetically rare phenotype or a limiting medical condition. Some patients (0.1–0.5 %) who are repeatedly transfused blood with products including RBCs can develop multiple antibodies against the RBC antigens. These alloimmunized patients need to be transfused with specific RBCs that lack those antigens. However, it is often very difficult to obtain adequately matched RBCs.

In other patients, for example patients with chronic anemia who require long-term transfusion, personalized RBC products would be necessary and much more valuable than natural blood from multiple donors, as use of personalized RBC products could minimize antigen exposure and the risk of blood-transmitted infections. In these patients, advantages of lab-grown personalized RBC products would outweigh the disadvantages of traditional donated blood products in spite of the higher cost of personalized RBC products. The priceless value could become possible by establishing iPSC cell lines.

For the last 5 years, there have been several studies regarding RBC production from iPSCs, and the production efficiency has been improved. Terminal erythroid differentiation and increased expression of fetal hemoglobin from iPSCs was reported by Lapillonne et al. (2010). However, the RBC yield is still too low, and the clinical efficacy and safety of iPSC-derived RBCs has not yet been tested.

## 20.5 "Blood Pharming": Opportunities and Challenges in RBC Manufacturing

In the years since this promising research began in 2004, several industries and governments have invested in research regarding large-scale blood manufacturing. For example, The US Defense Department in 2007 launched the "Blood Pharming" program, which aims to produce RBCs more quickly than can be achieved using BM through the use of a small bioreactor developed through a partnership with Arteriocyte Inc. (Cleveland, Ohio). They expect to be able to produce 20 units of RBCs in 3 days, but as of yet there have been no published reports regarding their results.

One of the most important issues for RBC production is how sufficient numbers of RBCs can be produced from small numbers of stem cells. One packed RBC unit has 2–4 $\times$ $10^{12}$ RBCs. More than 90 million units of whole blood are collected annually from donors worldwide. (www.who.int/worldblooddonorday/en/). To meet the huge demand for blood in routine clinical practices, automated continuous production systems using bioreactors are essential. More recently, several groups have developed a bioreactor system for RBC manufacturing from cord blood stem cells (Timmins et al. 2011; Housler et al. 2012). However, the expansion magnitude, the manufacturing efficiency, and the quality and functionality of generated RBCs must be improved further. In general, $10^5 \sim 10^6$ HSCs are manually or automatically isolated and inoculated at a scale 8 mL. The

process of automatic isolation is very costly and would be hard to be used in RBC manufacturing. In early stages, cells are still grown manually, which increases the chances of contamination and cannot guarantee stable results. In later stages, the current best technology in the bioreactor area allows for a maximum cell density of $2 \times 10^6$ cells/mL, meaning that at least 1,000 L of culture media are necessary for the production of one pack of RBCs (Timmins and Nielsen 2011). Currently available bioreactors are thus not sufficient to meet the demand for RBC production. Even though cell expansion and enucleation processes have been developed, there still remain several unsolved issues; i.e, how the huge final media volume at the final culture stage could be handled and how pure RBCs can be isolated. Creative approaches will need to be developed, but practical RBC production in vitro could be possible in the near future, just as many other problems have been solved in the stem cells and biotechnology areas.

Another critical hurdle is the high cost of industrial production of RBCs. The current theoretical cost to make one unit of RBC is estimated as 5,000 US dollars (Lippi et al. 2011). However, technical advances may facilitate this procedure and reduce costs to a reasonable level. Reducing costs to the level of donated blood products may not be feasible in the next 5 years. However, if cultured RBCs could be generated from donors with very rare blood types or could be produced automatically in isolated areas where blood supplies are not easily accessed, the value of manufactured blood would surpass the high cost of production.

## 20.6 Conclusions

The speed of development of industrially manufactured RBC products must be increased as a severe global blood supply shortage is anticipated to occur in the next several decades. In some countries, donors will be able to meet less than half of the demand by 2030–2050 as a result of the rapidly aging population. In addition, if outbreaks of pandemic infectious diseases occur, the lack of blood supply could be fatal to many.

A frequently used slogan in blood centers is “There is no option other than your blood donation.” Once blood manufacturing is available, people may not be inclined to give their blood for free, and donation levels may decline sharply. If that were to occur, much of the blood supply would need to be replaced by industry-made blood products.

It is unknown when the use of manufactured RBC components will be common in hospital blood banks. Inferring from the success of *in vitro* generation of RBCs in 2005 to its first human trial in 2011, which was relatively in short time, our goals may be reached within a decade. However, the clinical efficacy has not yet been fully tested. There are many ongoing clinical trials using stem cells. However, recent studies warned that the hESCs and iPSCs have showed genetic instability when they were cultured for a long time (Maitra et al. 2005; Laurent et al. 2011). Similarly, it is not possible to completely mimic nature and produce

perfect RBCs in vitro without side effects such as easily hemolyzed RBCs or tissue stress due to fetal-type RBCs with high oxygen affinity. However, once RBC manufacturing process from stem cells can be industrialized, the cultured RBCs will become the most widely used stem cell product as cultured RBC products are far superior to donated blood in terms of safety and less immune responses.

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# Part V
# Potential Applications of HBOCs

# Chapter 21
# Liposome-Encapsulated Hemoglobin: Potential Clinical Applications

**Akira T. Kawaguchi, Chieko Murayama, Fumiaki Yoshiba, Hiroyuki Furuya, Mariko Yamano and Munetaka Haida**

## 21.1 Introduction

Artificial oxygen carriers were originally developed as a substitute for red blood cells (RBC) for transfusion. As the blood demand–supply balance becomes critical, it is reasonable to explore artificial oxygen ($O_2$) carriers. Although synthetic $O_2$ carriers have long been studied (Nose 2004), nothing has yet satisfied their safety issues (Kano and Kitagishi 2009). In their stead, it is natural to use hemoglobin (Hb) as source and/or substrate of artificial oxygen ($O_2$) carriers, since Hb has been tested in vertebrates for millions of years. First, crude cell-free human Hb has been tested, but with immediate evidence of adverse events. Then, modified types of Hb or acellular Hb were developed and tried in clinical studies, during which all of them were associated with increased incidence of myocardial

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infarction and death, instead of prospective advantages compared with control patients receiving asanguineous solutions (Natanson et al. 2008). Moreover, recent studies (Koch et al. 2008) have disclosed that even human RBC becomes deleterious when used after 2 weeks of donation or later. Since these disadvantages appeared to be related to the inherent toxicity of free Hb (Simoni et al. 2009), this naturally suggests either association of nitric oxide (Kawaguchi et al. 2009) or encapsulation of Hb (Chang 1991; Sakai et al. 2009; Ogata 2000; Kaneda et al. 2009) to simulate the natural structure of RBC to preserve its function while preventing its toxicity.

## 21.2 History

In the 1950s Chang et al. were the first to develop liposome-encapsulated Hb (LEH) (Chang 1991), followed by the US Naval group. Tsuchida et al. of Waseda University (Sakai et al. 2009) and Terumo, Co., Ltd. (Ogata 2000) continued to develop and modify LEH to prolong in vivo half-life, to improve $O_2$ carrying capacity, and to prospectively realize its clinical utility. A recent breakthrough was the surface modification with polyethylene glycol to prevent removal from circulation by the reticuloendothelial system (RES) that destructs and metabolizes LEH as in the natural cascade of the RBC metabolism. As a result, the current LEH extends the intravascular retention half-life up to over 10 h in rodents and up to 70 h in nonhuman primates (Kaneda et al. 2009). The following is a summary of our experience with LEHs, which were manufactured and provided by Terumo, Co., Ltd. (Tokyo, Japan).

## 21.3 Characteristics

The size of the LEH particle (200–250 nm) is much smaller than that of RBC (Fig. 21.1). The nanometer size gives flow characteristics different from those of RBC (Urakami et al. 2009), a large surface area for the contained unit of Hb, and physical strength against the shear stress. Co-encapsulation of allosteric effectors makes it possible to modify $O_2$ affinity of LEH, varying from extremely high ($P_{50}O_2 = 10$ mm Hg) to extremely low ($P_{50}O_2 = 40-50$ mm Hg). The low $O_2$ affinity may theoretically increase $O_2$ delivery under room air respiration, and be even more efficacious when the recipients breathe supplemental $O_2$ (Fig. 21.2). It has been expected to reduce Hb requirement while maintaining comparable $O_2$ transport equivalent to RBC transfusion (Ogata 2000; Kaneda et al. 2009). This was expected to be most suitable for emergency transfusion to patients with hemorrhagic shock (Nogami et al. 2008; Yoshiba et al. 2009; Ikegawa et al. 2012). In contrast, a higher $O_2$ affinity may be advantageous for delivering $O_2$ to hypoxic tissues in case of organ ischemia/infarction (Kawaguchi et al. 2007, 2009, 2010, 2013; Fukumoto et al. 2009;

Okamoto et al. 2009; Okada et al. 2012; Kurita et al. 2012; Fukui et al. 2012) or radiotherapy to malignant tumor associated with an underdeveloped vascular system (Murayama et al. 2012).

## 21.4 As a Substitute for RBC

Although RBC transfusion is considered superior to anything else, LEH has been expected to be suitable in cases of hemorrhagic shock with unknown blood types (Nogami et al. 2008; Yoshiba et al. 2009; Ikegawa et al. 2012). No recognizable surface antigen on LEH (Fig. 21.1) allows administration without any blood-typing, screening for irregular antibodies, or cross-matching without concerns of major and/or minor mismatch transfusion. In an emergency transfusion for patients with unknown blood type, we have been using type-O Rh+ RBC transfusion (ER-Tx) at Tokai University Hospital to maintain hemodynamics and aerobic metabolism before identical RBC becomes available for transfusion (Yoshiba et al. 2009). In retrospect, those patients with compatible blood types who had minor mismatch transfusion had a similar survival rate to those who happened to have an identical blood type (O Rh+) (Fig. 21.3). Although the duration of time that could be spared would be 60 min at most, it is crucial for the prognosis in such patients. In 75 cases with exact time records of blood loss event and ER transfusion, 49 patients who received ER-Tx within 60 min of blood loss had a significantly better survival than 26 patients who received the same transfusion later (Yoshiba et al. 2009), suggesting that the earlier transfusion is better than the exact match. In the

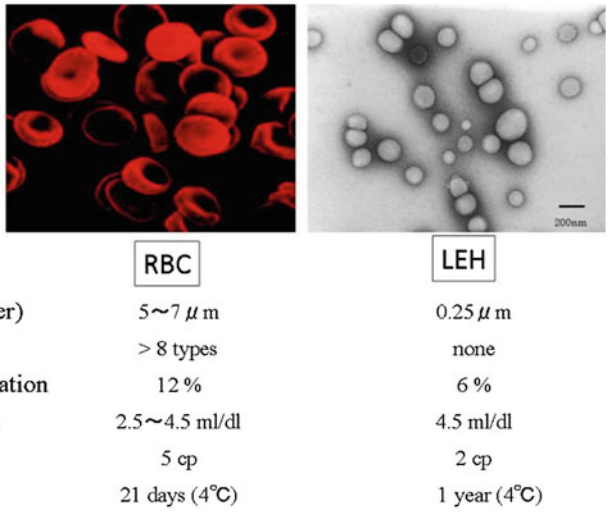


**Fig. 21.1** Characteristics of LEH

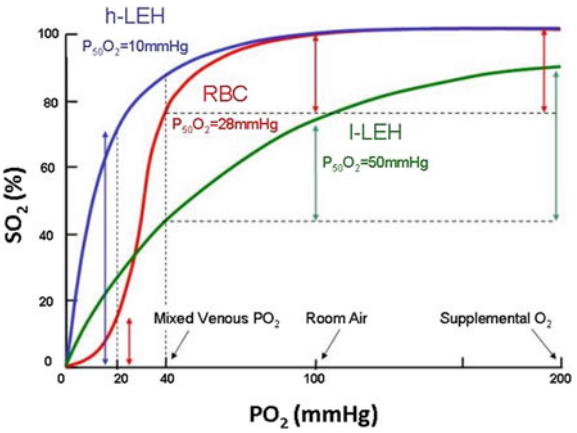


**Fig. 21.2** *Oxygen Affinity* $O_2$ binding characteristics of LEH with high $O_2$-affinity (h-LEH, $P_{50}O_2 = 10$ mm Hg), RBC, and LEH with low $O_2$-affinity (l-LEH, $P_{50}O_2 = 50$ mm Hg). While h-LEH (*blue arrow*) is assumed to deliver more $O_2$ than RBC (*red arrow*) under $PO_2$ between 20 and 0 mm Hg (hypoxic condition), l-LEH (*green arrow*) is presumed to deliver more $O_2$ than RBC (*red arrow*) under $PO_2$ between 200 (supplemental $O_2$ respiration) and 40 mm Hg (mixed venous $PO_2$)

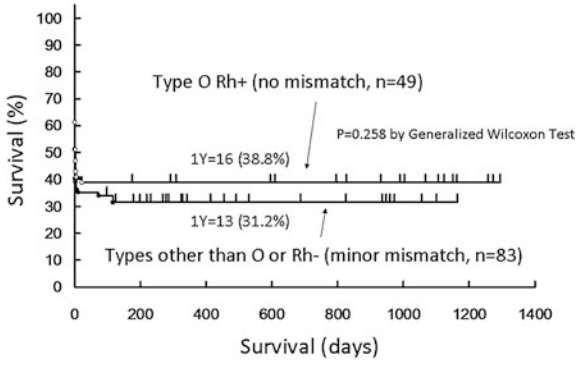


**Fig. 21.3** *ER-MAP and Survival* Among the initial 132 patients who received emergency ER-Tx (type O Rh+), there was no significant survival difference between 49 patients who happened to have blood type O Rh+ and 83 patients with other blood type

operating room, which has the largest requirement/usage of blood products, the use of LEH for the first 8 (200 mL-derived) units could drastically reduce routine pre-transfusion testing and matching to 7.4 % of the current transfusion practice, as most patients receive only a few units of transfusion (Yoshiba et al. 2009). Since a great deal of effort and meticulous attention have been expended to the delivery of blood as soon as possible after donation (Fig. 21.4), the introduction of LEH as an RBC substitute for transfusion may not only minimize transfusion-related risks but also reduce the work load and stress on manpower in the blood bank. In the current situation, prolonged shelf-life up to 1 year (Sakai et al. 2009) is especially

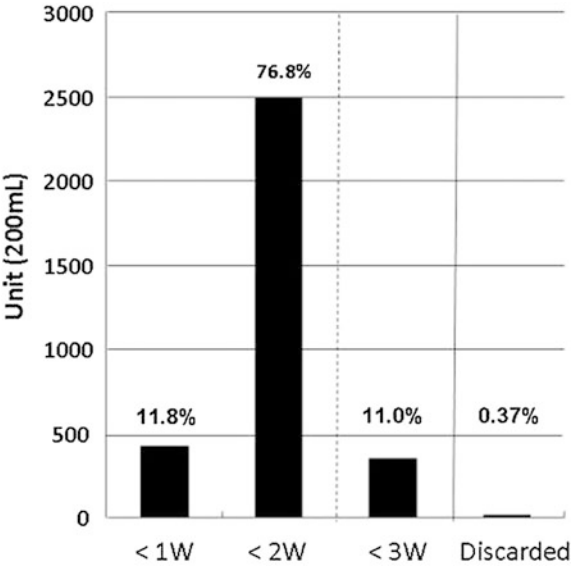


**Fig.. 21.4** *Use of Donated Blood at Tokai University Hospital* was studied during the month of February 2012. Most blood units (derived from 200 mL) were used within 2 weeks of donation, and the discarding rate (aged over 3 weeks after donation) was kept to 0.37 % by meticulous efforts in the blood bank

advantageous for preparation or stock in medical facilities, reducing the necessity for routine replacement of aged RBC, resulting in reduced discard of expired RBC, which may then be recycled to the production of LEH.

## 21.5 Safety Issues

The risk of mismatch transfusion and contamination could be minimized because LEH is manufactured from purified human Hb under strictly sterile conditions. Besides these biological risks associated with human-derived factors, there are substantial concerns related to the metabolism of LEH in vivo. Whereas LEH in itself was found to be inert in a mouse model reconstituted with human cord blood (Kawaguchi et al. 2009c), LEH had been reported to reduce phagocytotic activity, which may suppress the initial immune response of the recipient to 3rd party antigens. A hemorrhagic shock patient requires emergency transfusion due largely to accidents or surgery, when the patient may often be concurrently exposed to infectious organisms or tumor cells. Moreover, it is likely that emergency infusion of LEH may make the situation worse because its half-life is much shorter than that of RBC. If a large amount of LEH occupies or overwhelms the metabolic capacity of RES, relative suppression and delay in the initial antigen recognition process of the 3rd party antigen might allow establishment of infection or tumor dissemination. This concern was experimentally tested using a model of transgenic mice (Kawaguchi et al. 2012), which disclosed that the initial antigen recognition

process remains intact immediately after the administration of LEH at 20 mL/kg, equivalent to the amount of ER-Tx for patients with hemorrhagic shock at Tokai University Hospital (Yoshiba et al. 2009).

## 21.6 As an Oxygen Therapeutic

Instead of its utility as a substitute for transfusion, where RBC is undoubtedly the best option, characteristics of LEH make it useful under various pathologic conditions where RBC is not useful or therapeutic. As stated above (Fig. 21.1), the particle size of LEH is so small that its perfusion pattern is not like RBC but rather like plasma (Urakami et al. 2009; Kawaguchi et al. 2007; Fukui et al. 2012). Since plasma has been reported to perfuse to the core of cerebral ischemia (Theilen et al. 1994), LEH may deliver $O_2$ in case of ischemia, preserve the aerobic metabolism in hypoxic tissue, and result in reduced reperfusion injury and its sequellae. The capability of modifying $O_2$-affinity may further increase the amount of $O_2$ delivery to hypoxic tissues (Fig. 21.2). We have been exploring this hypothesis in ischemia/reperfusion and/or circulatory derangement in various tissues and organs in the following experimental settings.

### *21.6.1 Cerebral Ischemia and Reperfusion*

Cerebral ischemia is the most important emergency situation, since reperfusion time allowance is limited to less than 3 h (Adams et al. 2007). LEH was first tested in permanent occlusion of the middle cerebral artery (MCA) in the rat (Kawaguchi et al. 2009). LEH administration significantly reduced cerebral edema and infarction as detected by magnetic resonance imaging (MRI), showing that its protection extended not only to the lobes of the ischemic hemisphere but also to the contralateral hemisphere (Kawaguchi et al. 2009). The protective effect on cerebral reperfusion was then examined in a photochemically-induced MCA occlusion model in the rat (Kawaguchi et al. 2007), where the dose–response relationship and a comparison between high and low $O_2$-affinities of LEH were examined. Surprisingly, the dose–response relationship was not linear but convex, with 2 mL/kg the most efficacious dose and 0.4 mL/kg the minimum effective dose. Increasing the LEH dose to 10 mL/kg failed to yield increased protection. This phenomenon was also true in subsequent experiments using nonhuman primates, which were conducted to simulate the current clinical cerebral reperfusion therapy: 3-h MCA occlusion and reperfusion using positron emission tomography (PET) to examine the precise $O_2$ metabolism (Kawaguchi et al. 2010). Administration of LEH increased radioactive $[^{15}O]O_2$ in the plasma fraction in a dose-dependent manner (Fig. 21.5). Nonetheless, 2 mL/kg was found to be most protective, and increasing the dose to 10 mL/kg also failed to improve the benefit

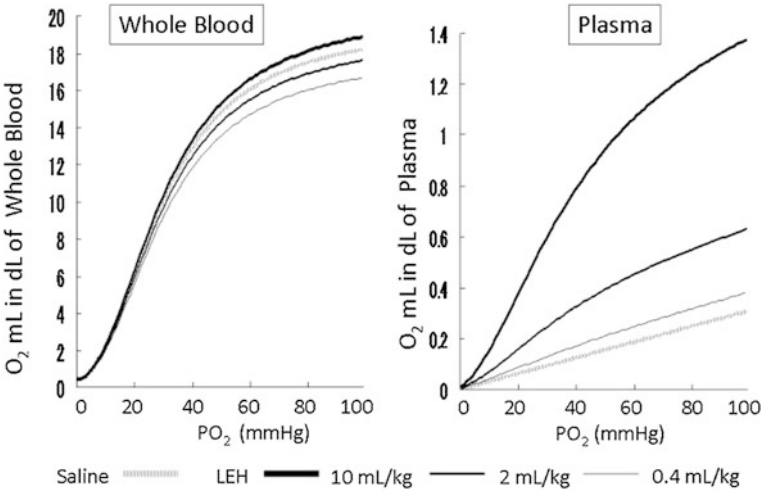


**Fig. 21.5** *Calculated $O_2$ Content During MCA Occlusion* The $O_2$ content was calculated based on Hb from measured RBC volume (*hematocrit*) and LEH volume during cerebral ischemia in monkeys undergoing 3-h MCA occlusion and reperfusion study (Kurita et al. 2012). Various treatments 5 min after MCA occlusion did not make much difference in $O_2$ content in whole blood, whereas there was a significant and dose-dependent difference in $O_2$ content in the plasma fraction

in this model. $O_2$ metabolism in ischemic brain tissue was not different between the treatment groups during ischemia and up to 3 h after reperfusion, when the cerebral metabolic rate of $O_2$ ($CMRO_2$) was preserved in the cortex of animals that received LEH 5 min after onset of MCA occlusion. In this model, basal ganglia were more severely damaged by ischemia and/or reperfusion injury (Fig. 21.6) regardless of the presence or absence of LEH (Kawaguchi et al. 2013). Although LEH administration significantly preserved the cortex, it failed to extend any protection to basal ganglia. Such difference in vulnerability has been recognized in the rodent models as well (Kawaguchi et al. 2007, 2009; Fukumoto et al. 2009). The contrasting images of LEH deposition (Fig. 21.7) in the infarcted tissues where MAP2 was depleted and vice versa suggested that LEH leaked through the ischemia-induced malfunction of the blood brain barrier and deposited in the damaged tissue while LEH in the circulation perfused the tissue repeatedly to supply $O_2$ to support the aerobic metabolism. Such benefit of LEH treatment in acute phase after cerebral ischemia/reperfusion was found to persist for up to 8 days (Kawaguchi et al. 2013), when glucose as the cerebral metabolic substrate showed a similar pattern of metabolism or protection only in the cortex. Precisely, however, preserved $CMRO_2$ in the cortex was due to the increased $O_2$ extraction fraction (OEF), not recovered cerebral blood flow (CBF), suggesting that the cortex remains partly ischemic 8 days after the ischemia/reperfusion event. This was also associated with increased $CMRO_2$ in the contralateral hemisphere, suggesting a compensatory mechanism to support functional recovery, not association

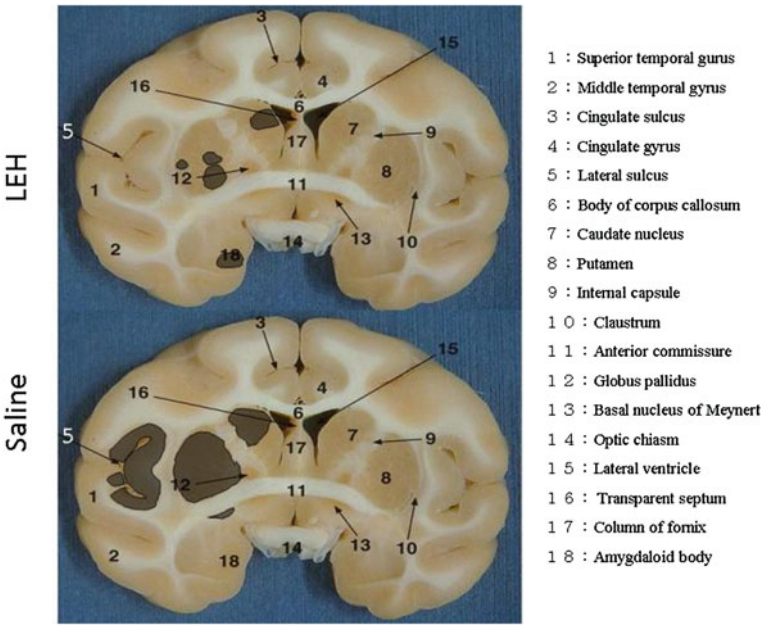


**Fig. 21.6** *Gross Brain Anatomy* A frontal cross-sectional brain anatomy was shown in monkeys treated with LEH (2 mL/kg) and saline (2 mL/kg) 8 days after MCA occlusion and reperfusion (Fukui et al. 2012). The macroscopic infarction was marked in brown, showing the distribution and area of infarction. Whereas LEH treatment restricted the damage mainly to basal ganglia (*upper panel*), saline-treatment resulted in large infarcted areas in basal ganglia and adjacent cortex, or lateral sulcus (*lower panel*)

with morphological restoration. Basal ganglia were associated with decreased $CMRO_2$ due to reduced OEF and CBF, indicating a complete infarction (Kawaguchi et al. 2013) (Figs. 21.6, 21.7).

## *21.6.2 Cochlear Ischemia and Reperfusion*

Cochlear ischemia has been considered to be the leading cause of sudden deafness, a common disease that potentially leads to serious auditory defects unless appropriately treated. Since the cochlear artery is an end-artery with no other collaterals, a spasm may easily lead to hearing defects as seen in clinical cases. Such pathology was modeled with use of the Mongolian gerbil that lacks the posterior communication artery, so bilateral vertebral artery occlusion leads to a total hindbrain ischemia. In this model, pretreatment (Okada et al. 2012) showed a clear protective effect, with a significant difference between treatments with LEHs of different $O_2$-affinities, as LEH with high $O_2$-affinity (h-LEH) was more protective than LEH with low $O_2$-affinity (l-LEH), as reported by Fukumoto et al. (Kawaguchi et al. 2007); 1/5–1/25 dose of h-LEH worked as well as l-LEH. Since pre-ischemic

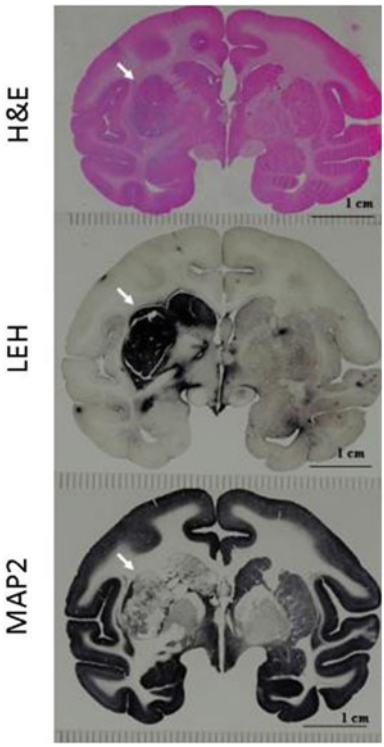


**Fig.. 21.7** *Histological Staining for H&E, LEH and MAP2* Three consecutive frontal cross-sectional slices stained by hematoxylin-eosin (*H&E*), immunohistochemical staining for human Hb (*LEH*) and for MAP2 (*MAP2*) in a monkey treated with LEH (2 mL/kg) 6 h after MCA occlusion and 3 h after reperfusion (Kurita et al. 2012). Basal ganglia (*arrows*) were largely infarcted, as demonstrated by the heavy deposition of LEH and depletion of MAP2 expression

administration is not clinically relevant, therapeutic use was examined in the same gerbil model [in preparation], revealing that LEH was still significantly effective, although less protective, than when it was administered prior to ischemia. Morphologic observation 7 days after ischemia/reperfusion showed that the internal hair cells, but not the outer hair cells, were lost sporadically regardless of pre- and post-ischemic treatment with saline or RBC, suggesting apoptosis rather than necrosis as the cause of deafness and cell death. This observation was quite unexpected, since the mechanism of action of LEH could be different from the original hypothesis; the protection afforded by LEH long after the ischemia/reperfusion event may still be related to improved microcirculation under increased intracochlear pressure as a mechanism(s) accounting for the benefits of LEH, or may be more related to another mechanism(s) that would suppress apoptosis of the internal hair cells, the sensory terminals of auditory neurons [Okada et al., in preparation].

### *21.6.3 Skeletal Muscle Ischemia and Reperfusion*

A rat model was developed to study intracellular energy metabolism using $^{31}P$ nuclear magnetic resonance (NMR) in skeletal muscular ischemia and reperfusion

(Kurita et al. 2012). This was modeled on the entrapment syndrome, which has been reported after major earth quakes. Because the skeletal muscle cell has affluent substrates, energy production as well as storage system, monitoring intracellular pH, inorganic phosphate (Pi) and high-energy phosphate (PCr) is of interest for determining the real-time effects of LEH in skeletal muscle ischemia and reperfusion. LEH administration caused an immediate change in intracellular pH, which decelerated progressive intracellular acidosis during administration, showing a significant difference from that of animals treated with saline, RBC, or non-treated. In contrast, PCr/Pi, an index of the intracellular energy status, decreased precipitously regardless of treatment after ischemia, while it increased significantly after reperfusion only in animals treated with LEH. Since anaerobic degradation of PCr is a common step of the muscle to develop ATP, abrupt depletion of $O_2$ supply may cause a similar intracellular energy metabolism to that detected in PCr/Pi reduction. In contrast, intracellular pH depends greatly on the dynamic balance between the production and washout of lactate, and LEH might be effective in supplying $O_2$ to preserve aerobic metabolism, to effectively suppress lactate production and eventually decelerate the progression of intracellular acidosis. This environment might have preserved capillary perfusion and recovery of PCr/Pi after reperfusion, while rats with no infusion showed no improvement in PCr/Pi during the experimental period. These metabolic observations appeared to be due to thrombotic occlusion of arterioles, most prominent in animals with no infusion, in histological findings 7 days later.

### *21.6.4 Surgical Wound Healing*

The application considered first for LEH in surgery was wound healing after gastrointestinal (GI) operation, since dehiscence seriously complicates recovery of patients but there is no way to establish a blood supply. Thus, the pathological background is different from ischemia/reperfusion injury, as a postoperative wound has deranged circulation, namely, a severed, discontinued microcirculation network. Since GI tract function is difficult to detect, bursting pressure of the wound was used as a surrogate of the level of wound healing. LEH was found to accelerate wound healing 2 days after surgery in a rat gastric suture model (Okamoto et al. 2009). However, 4 days later its effects were no longer significant, as the other treatments groups had caught up in terms of bursting pressure. Histopathologically, there was significantly less neutrophil infiltration and more macrophage appearance compared to the other groups treated with RBC or empty liposome at 2 days post surgery. Two days later, or 4 days post surgery, these differences disappeared, as with the bursting pressure. Since the rats treated with empty liposome acted just like the animals receiving RBC, these differences were considered to be due to the human Hb in the LEH, which is xenogeneic and attracts rodent macrophages. The dose–response relationship showed that as little as 0.4 mL/kg of h-LEH was significantly effective compared to saline treatment [in

preparation]. To determine the mechanism of action, we examined the frequency of hypoxia inducible factor 1 alpha (HIF-1α) positive cells in anastomotic tissue, which had been suppressed only in the animals treated with h-LEH starting from 6 h up to 48 h post surgery, when the anastomotic strength was significantly higher than in the other treatment groups [in preparation]. Thus, we believe that LEH administration at the time of GI surgery may somehow suppress the inflammatory response and accelerate wound healing by preserving aerobic metabolism at the anastomotic site at least for the first 48 h. After that, the natural healing process would start to catch up with the LEH treatment by 4 days after surgery.

### *21.6.5 Skin Defect Healing*

Other than wound healing in surgery, the healing problems include decubitus (pressure perfusion derangement) and diabetic healing delay. We tested the effects of LEH in a mouse skin defect or ulcer model (Fukui et al. 2012). Each mouse had two skin defects (ulcers) on the back on Day 0. On Day 2, mice with similarly-sized ulcers were grouped together, and they received saline and Hb at amounts that equaled those of RBC or LEH. The magnitude of the healing process was measured in the area of the ulcer in terms of a silicone ring holding the skin back from contraction, which showed reduction of the ulcer being facilitated by the LEH treatment compared to the other treatments as early as 2 days after the 1st dose (2nd dose, Day 4), and persisting 3 days later (Day 7). While Laser-Doppler flowmetry showed no difference between treatments, there was a significant histological difference in the development of granulation and epithelialization. Similar to the observation in the gastric wound, there was significantly less neutrophil infiltration at 2 days after the 1st treatment (Day 4), but not 3 days later (Day 7), when signs of inflammation had subsided in all groups. Immunohistochemical study disclosed significantly increased Ki67 positive cells in LEH-treated mice on Day 4 as well as on Day 7, suggesting increased tissue regeneration parallel to a reduction in ulcerous area.

## 21.7 Sensitization of Cancer Radiotherapy

Underdevelopment of the vascular system makes cancer tissue hypoxic and resistant to radiotherapy. Therefore, delivering $O_2$ to malignant tissue may intensify radiation therapy. We tested this long-standing concept by pretreatment with h-LEH (Murayama et al. 2012). Mice implanted with squamous cell carcinoma cells received various doses of h-LEH 30 min before irradiation (20 Gy). When subsequent tumor growth was monitored for measuring the efficacy of combined h-LEH and radiotherapy (Fig. 21.8), administration of LEH (10 mL/kg) 30 min before irradiation was most effective in suppressing the following tumor

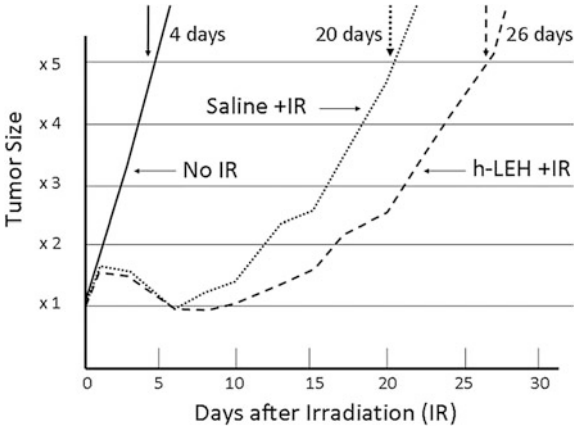


**Fig. 21.8** *Tumor Growth Delay* Tumor growth was plotted against time (*day*) after various intravenous treatments and irradiation therapy (IR, 20 Gy). While radiation suppressed the tumor growth, pretreatment with h-LEH (10 mL/kg) shifted the growth curve further to the right (the vertical arrow depicts that 5 times growth took 26 *days*), compared to saline (the vertical arrow indicates that 5 times growth took 20 *days*), suggesting significant sensitization of radiotherapy by h-LEH pretreatment

growth. However, the impact of the sensitization was not persistent, with 30 and 120 min before irradiation being effective while 90 min not differing from empty liposome-treated control animals. Deposition of h-LEH was apparent 30 min after administration, increasing up to 24 h and decreasing thereafter. This was parallel to the suppression of HIF-1α expression in the tumor, where HIF-1α was suppressed from 6 h after h-LEH administration and thereafter (Fig. 21.9). These observations suggest that h-LEH may help increase $O_2$ tension in the tumor to improve the effects of radiotherapy (Murayama et al. 2012). Although precise mechanism(s) remains unknown, this experiment supports another ongoing study to examine whether h-LEH might intensify tumor chemotherapy as well, since exposure time and duration are much longer than with radiation therapy (Murayama, in preparation).

## 21.8 Prospects

All the information so far may suggest the safety and advantage of encapsulation to separate Hb from the vascular wall, similar to the mechanism and structure of RBC. Since the biological and/or immunological response to xenogeneic Hb is not clear at this time, it is necessary to manufacture LEH from human Hb, which so far remains the most serious limiting factor. Whereas 0.4–2 mL/kg of LEH would require 24–120 mL per person (60 kg) as a therapeutic, the amount required for RBC substitute for emergency transfusion may be as much as 814 mL (Yoshiba et al. 2009),

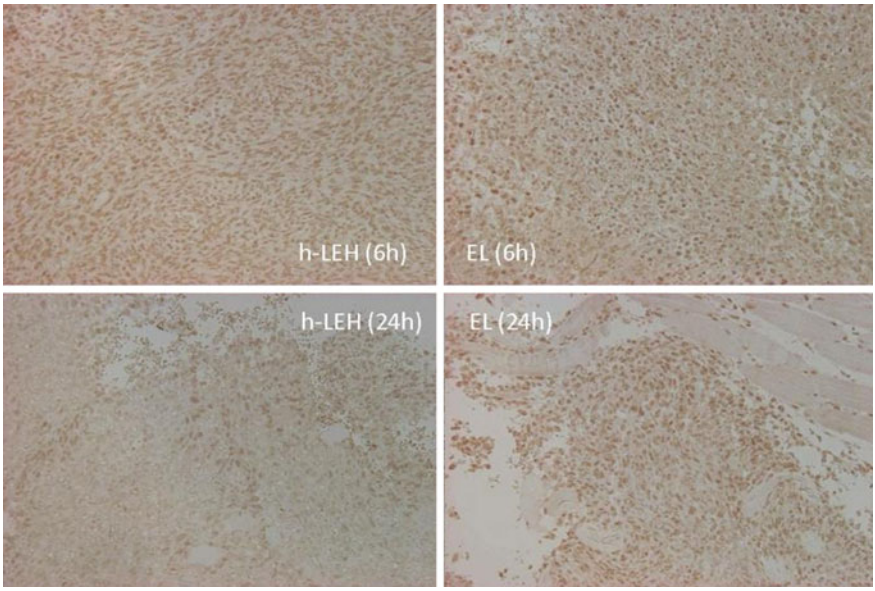


**Fig. 21.9** *Immunohistochemical Staining for HIF-1α after h-LEH* Immunohistochemical staining for HIF-1α in squamous cell carcinoma after intravenous h-LEH (*left panels*) or empty-liposome treatment (EL, *right panels*) to the host mice. Although HIF-1α deposition was equivalent 6 h after the treatment (*upper panels*), most tumor cells remained positive for HIF-1α 24 h after EL treatment, whereas most tumor cells remained negative for HIF-1α 24 h after h-LEH treatment

which remains difficult to develop de novo by the current technology. It is expected that gene technology may soon provide human RBC and Hb as the source of transfusion and substrates for LEH production. Until that time, it seems necessary to depend on donated blood for transfusion, as for organ transplantation, and as the sources of human Hb for manufacturing LEH as an artificial organ. Both of them will have requirements and clinical utility to complement each other to improve clinical outcomes, a therapeutic combination like organ transplantation and artificial organs as the treatment for end-stage organ failure such as the kidney as well as the heart.

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# Chapter 22
# Biocompatibility of Hemoglobin Vesicles, a Cellular-Type Artificial Oxygen Carrier, on Blood Cells and Plasma Proteins In Vitro and In Vivo

**Hiroshi Azuma, Mitsuhiro Fujihara and Hiromi Sakai**

## 22.1 Introduction

Vigorous efforts have been undertaken to develop hemoglobin (Hb)-based oxygen carriers (HBOCs) for use as red blood cell substitutes (Sakai et al. 2008). HBOCs present several potential benefits for red blood cell transfusion applications, including the absence of blood-type antigens and infectious viruses and the ability to be stored stably for long time periods. HBOCs are expected to satisfy emergency purposes until allogeneic transfusion of compatible red cells. Moreover, their use can satisfy requirements for huge amounts of red cells in times of catastrophe. Consequently, HBOCs can contribute to construction of an ideal blood program when used in conjunction with present allogeneic transfusion capabilities.

HBOCs are categorized into two types: acellular modified Hb molecules and cellular liposome-encapsulated Hb. Actually, hemoglobin-vesicles (HbV, developed by Waseda University) are of the latter type. They have phosphatidylcholine, cholesterol, PEG-conjugated lipid, a negative charged lipid and concentrated Hb molecules, as do actual red blood cells (Sakai et al. 1997). Their sufficient $O_2$ transport capability, comparable with that of blood, has been established in several animal models (Sakai et al. 2008). The distribution of HbV after administration and the prompt metabolism of HbV in the reticuloendothelial system have been demonstrated (Sakai et al. 2001; Sou et al. 2005).

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The biocompatibility of HbV is an important issue for the clinical use of these materials. Several indexes of biocompatibility have been proposed. In this chapter, we present biocompatibility of HbV for human blood cells and human plasma proteins in vitro and in immune systems in rat models.

### *22.1.1 Effect of HbV on Human Platelet Function*

Circulating platelets bind to the subendothelial matrix of injured vessels and subsequently become activated, causing the release or the expression of components in their intracellular granules and the formation of metabolic products. These products include prothrombotic substances (e.g., adenine nucleotides, thromboxane $A_2$ [$TXA_2$], serotonin and CD62P) (Rand et al. 2003) and an array of potent proinflammatory chemokines (e.g., RANTES, MIP-1) (Gawaz et al. 2005). Prothrombotic substances function as agonists for the recruitment of additional platelets into the evolving thrombus. Chemokines released from the activated platelets trigger the recruitment of leukocytes into the evolving thrombus and play a large role in the initiation and perpetuation of inflammatory responses (Baggiolini and Dahinden 1994).

Platelet activation is apparently necessary to prevent bleeding in vivo. However, nonphysiological activation engenders pathological thrombosis and the modulation of inflammatory responses. The biocompatibility of HbV and human platelets was evaluated by examining the effects of HbV on the most frequently used platelet activation markers (i.e., CD62P expression and the binding of activation-dependent $\alpha_{IIb}\beta_3$ antibody PAC-1 to platelets) in the presence or absence of agonists in vitro. We also investigated the effects of high concentrations of HbV (up to 40 %) on the secretion of other substances (i.e., serotonin, RANTES, and $\beta$-thromboglobulin [$\beta$-TG]) and the formation of thromboxane $B_2$ ($TXB_2$), a metabolite of $TXA_2$.

In this series of experiments, our earlier formulation of HbV containing DPPG (DPPG-HbV) and the present formulation of HbV containing a different type of negative charged lipid, 1, 5-O-dihexadecyl-N-succinyl-L-glutamate (DHSG) (DHSG-HbV) were used. Table 22.1 presents our results, demonstrating that incubation of human platelets to high concentrations of HbV in vitro did not cause platelet activation. Moreover, it did not adversely affect the formation or secretion of prothrombotic substances or proinflammatory substances in response to platelet agonists. Although a marginal reduction of spontaneous release of RANTES by HbV and a slight potentiation of ADP-triggered PAC-binding in the presence of HbV were noted, these effects were regarded as less meaningful from a clinical perspective (Wakamoto et al. 2001, 2005). Results show that HbV has superior biocompatibility to human platelets.

**Table 22.1** Effect of HbV on human platelets

| Index | Stimulant | Type of HbV | Conc. of HbV (%) | Effect |
|---|---|---|---|---|
| RANTES | Collagen (+) | DPPG-HbV | ≤20 | No effect |
| Release | (−) | DPPG-HbV | ≤20 | No effect |
| RANTES | Collagen (+) | DHSG-HbV | ≤40 | No effect |
| Release | (−) | DHSG-HbV | ≤40 | Marginal reduction |
| $\beta$-TG Release | Collagen (+) | DHSG-HbV | ≤40 | No effect |
| | (−) | DHSG-HbV | ≤40 | No effect |
| Serotonin | Collagen (+) | DHSG-HbV | ≤40 | No effect |
| Release | (−) | DHSG-HbV | ≤40 | No effect |
| $TXB_2$ | Collagen (+) | DHSG-HbV | ≤40 | No effect |
| Production | (−) | DHSG-HbV | ≤40 | No effect |
| CD62 | ADP (+) | DHSG-HbV | ≤40 | No effect |
| Expression | (−) | DHSG-HbV | ≤40 | No effect |
| PAC-1 | ADP (+) | DHSG-HbV | ≤40 | Slight potentiation |
| Binding | (−) | DHSG-HbV | ≤40 | No effect |

(cited from reference Fujihara et al. (2008))

### *22.1.2 Effects of HbV on Neutrophil Functions*

Neutrophils play important roles on host defense against various infectious agents. Neutrophils perform various functions (e.g., chemotaxis, superoxide generation) in response to zymosan as well as bacterially derived peptides such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Zu et al. 1998). A certain type of liposome modified with PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) was reported to reduce chemotaxis in response to these agents (Hatipoglu et al. 1998). In contrast, liposomes composed of phosphatidylcholine and phosphatidylserine have been shown to recruit neutrophil in the lungs of allergic-model mice (Bellemare et al. 1995). The interaction of HbV and neutrophils is apparently important in terms of the biocompatibility of HbV. With this in mind, we evaluated effects of HbV on four major functions of human neutrophils in response to fMLP (Table 22.2).

The results of the earlier formulation of HbV containing DPPG (DPPG-HbV) are shown in Table 22.2. Pre-incubation with HbV did not affect fMLP-triggered chemotaxis, upregulation of CD11b expression, degranulation of gelatinase granules (gelatinase-B), or superoxide generation under those experimental conditions (Ito et al. 2001). Consequently, the composition of phospholipid in HbV

**Table 22.2** Effect of DPPG-HbV on fMLP-induced neutrophil function

| Function | Conc. of HbV (%) | Effect |
|---|---|---|
| Chemotaxis | ≤0.6 | No effect |
| Upregulation of CD11b expression | ≤0.6 | No effect |
| Degranulation of gelatinase B | ≤6 | No effect |
| Superoxide production | ≤6 | No effect |

(cited from reference Fujihara et al. (2008))

neither suppresses nor activates the fMLP response of human neutrophils, which makes it highly biocompatible with neutrophils.

### 22.1.3 *Effects of HbV on Human Hematopoietic Stem/Progenitor Cells*

The large amounts of liposomes infused intravenously have been shown to distribute into the mononuclear phagocytic system (MPS) including Kupffer cells in the liver and macrophages in the spleen and bone marrow (Torchilin 2005). A radiolabeling study revealed that HbV administered intravenously distribute mainly to the liver, spleen and bone marrow (Sou et al. 2005). Concern has arisen over whether the HbV which are distributed into bone marrow might adversely affect hematopoiesis because the bone marrow is the major site of hematopoiesis. From this perspective, rats that received an acute 40 % exchange-transfusion with HbV showed complete recovery of hematocrit within 7 days because of elevated erythropoietic activity (Sakai et al. 2006). Furthermore, the number of red blood cells, leukocytes and platelets remained unchanged for 1 week after the infusion of HbV at 20 % of the whole blood volume. Findings obtained in these animal models strongly suggest the absence of inhibitory activity of HbV against hematopoiesis. However, the influence of HbV on human hematopoietic stem/progenitor cells has not yet been studied. We sought to evaluate the effect of HbV on the proliferation and differentiation of both the erythroid and myeloid lineages of cord blood (CB)-derived hematopoietic cells in liquid culture (Yamaguchi et al. 2009a).

As shown in Table 22.3, the incubation of HbV with CB-derived $CD34^+$ cells for up to 3 days had less effect on the proliferation of erythroid lineage ($CD235a^+$ cells) and myeloid lineage cells ($CD15^+$ cells). Furthermore, the incubation of HbV with CB-derived $CD34^+$ cells for up to 3 days had no adverse effect on the clonogenic activity of CB-derived hematopoietic cells (data not shown).

**Table 22.3** Effect of DHSG-HbV on the proliferation of erythroid and myeloid lineage cells in liquid culture

| Exposure period to HbV | $CD235a^+$ cells | | | $CD15^+$ cells | | |
|---|---|---|---|---|---|---|
| | HbV conc. (%) | | | HbV conc. (%) | | |
| | 0.75 | 1.5 | 3.0 | 0.75 | 1.5 | 3.0 |
| 20 h | 93.7 ± 10.0 | 94.9 ± 1.2 | 92.2 ± 8.8 | 100.8 ± 14.3 | 96.3 ± 7.9 | 96.6 ± 13.3 |
| 3 days | 85.2 ± 22.3 | 92.6 ± 11.5 | 89.0 ± 14.5 | 92.9 ± 6.1 | 95.8 ± 5.4 | 91.7 ± 4.7 |

Various concentration of HbVs were added to the medium containing the cord blood-derived $CD34^+$ cells. After 10 days' incubation, $CD235a^+$ cells for erythroid lineage and $CD15^+$ cells for myeloid lineage, respectively, were analyzed by flow cytometry. The number of $CD235a^+$ cells or $CD15^+$ cells at each concentration of DHSG-HbV is expressed as a percentage of the number in the control (HbV 0 %). Data are represented as the mean ± SD from three experiments performed on three separate cord blood donors. (cited from reference Fujihara et al. (2008))

We previously established a coculture system of human telomerase catalytic subunit-transfected bone marrow stromal cells and $CD34^+$ cells in vitro, by which the expansion of human hematopoietic stem/progenitor cells is visible. Using this in vitro expansion system, we found that the incubation of HbV with CB-derived $CD34^+$ cells up to 3 days had no adverse effect on the expansion of CB-derived hematopoietic stem/progenitor cells (data not shown) (Yamaguchi et al. 2009b). Taken together, the evidence shows that HbV are apparently biocompatible with human CB-derived hematopoietic stem/progenitor cells.

### *22.1.4 Effects of HbV on Complement Systems, Coagulation and the Kallikrein–Kinin Pathway in Human Plasma*

Negatively charged liposomes activate complements via both classical and alternative pathways in rat and human models (Chonn et al. 1991; Cunningham et al. 1979; Devine et al. 1994). Consequently, the reticuloendothelial system rapidly removes opsonized liposomes from blood circulation. Furthermore, complement activation can engender cardiovascular and pulmonary adverse responses, called complement activation-related pseudoallergy (CARPA) (Szebeni 2005; Szebeni et al. 2000). Indeed, certain types of liposome-encapsulated hemoglobin cause CARPA in pigs (Szebeni et al. 1999).

A negatively charged surface also triggers intrinsic coagulation pathway and the kallikrein–kinin cascade by activating coagulation factor XII (Griep et al. 1985; Mitropoulos et al. 1989). PEGylation was regarded as effective for prevention of complement activation by liposomes (Bradley et al. 1998; Klibanov et al. 1990; Woodle and Lasic 1992).

We evaluated the interaction of HbV between human plasma using HbV of three types: PEGylated HbV having DHSG (DHSG-HbV), PEGylated HbV having DPPG (DPPG-HbV) and DPPG-HbV without PEGylation (DPPG-HbV (no PEG)) (Abe et al. 2007). Coatsome EL-A was used as a highly negative-charged liposome without PEGylation. The EL-A greatly reduced the complement titer, but DHSG-HbV had no effect (Table 22.4).

**Table 22.4** Consumption of complement by HbV and liposome

| Additive | CH50 (U/mL) | |
|---|---|---|
| | (additive: serum) | |
| | 20:80 | 40:60 |
| Saline | 33.4 ± 2.8 | 21.4 ± 1.7 |
| DHSG-HbV | 33.5 ± 2.9 | 22.9 ± 2.4 |
| EL-A | 25.1 ± 2.7* | 5.9 ± 0.7* |

The complement titer (CH50) was measured using a 50 % hemolysis assay with a commercial kit. DHSG-HbV, saline or Coatsome EL-A (a negative-charged liposome) were mixed with serum at the indicated ratio (v/v) at 37 °C for 24 h. The lipid composition (mol %) of coatsome EL-A was DPPC:CHOL:DPPG = 30:40:30. Data are presented as mean ± SD using sera from five individuals. The CH50 of 100 % serum was 38 ± 3.2 U/mL. *$p < 0.05$ versus saline (cited from reference Fujihara et al. (2008))

**Table 22.5** Consumption of complement by various types of HbV

| Additive | CH50 (U/mL) (additive: serum) | |
|---|---|---|
| | 20:80 | 40:60 |
| Saline | 36.4 | 27.9 |
| DHSG-HbV | 37.6 | 31.4 |
| DPPG-HbV | 35.9 | 28.4 |
| DPPG-HbV (no PEG) | 29.9 | Under detection limit |

Complement titer (CH50) was measured using a 50 % hemolysis assay using a commercial kit DHSG-HbV, DPPG-HbV, DPPG-HbV (no PEGylation) or saline was mixed with serum as indicated ratio (V/V) at 37 °C for 24 h. The CH50 of 100 % serum was 45.1 U/mL (cited from reference Fujihara et al. (2008))

Among the three types of HbV, DHSG-HbV and DPPG-HbV show no reduction of the complement titer, although DPPG-HbV (no PEG) showed drastic reduction (Table 22.5).

In terms of coagulation activity, DHSG-HbV had no effect on the prothrombin time (PT) or on the activated partial thromboplastin time (APTT), but DPPG-HbV and DPPG-HbV (no PEG) tended to shorten APTT (data not shown). Furthermore, DHSG-HbV did not cause activation of the kallikrein–kinin cascade even when DHSG-HbV was mixed with plasma at 60 %, whereas DPPG-HbV (no PEG) and DPPG-HbV caused activation of the kallikrein–kinin cascade, producing a digested product. Collectively, DHSG-HbV, which is PEGylated HbV of the most advanced type, is highly biocompatible with human plasma protein.

### *22.1.5 Effects of HbV on Complement and Anaphylactic Reactions in Rats*

CARPA represents a novel subcategory of acute (type I) hypersensitivity reactions (HSR), which are mostly mild, transient and preventable using appropriate precautions (Szebeni et al. 2011). However, in an occasional patient, it can be severe or even lethal. Because a main manifestation of complement activation is cardiopulmonary distress, CARPA might be a safety issue primarily in cardiac patients. Although PEGylation is regarded as effective for prevention of complement activation by liposome, clinical experience shows that even PEGylated liposomal anti-cancer drug caused CAPRA, suggesting that PEGylation is insufficient to escape from the complement system in vivo (Laing et al. 1994; Laverman et al. 2001; Chanan-Khan et al. 2003). Therefore, we evaluated whether the infusion of HbV into rats affects the complement titer in vivo (Abe et al. 2007).

A transient decrease of the complement titer of the rat serum was apparent 3 days after the infusion of HbV or empty vesicle without hemoglobin (EV) (Fig. 22.1).

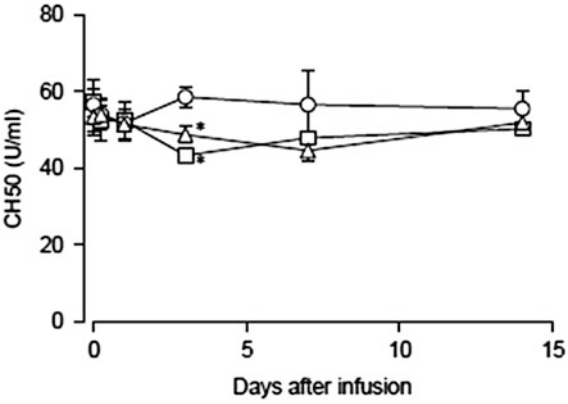


**Fig. 22.1** Changes of the complement titer in the rat serum after HbV infusion. HbV (*triangles*), EV (*squares*) or saline (*circles*) was infused into the rats at top-load from the tail vein. The complement titer in the rat serum was measured. It is shown as CH50. $N = 3$–4, mean ± SD $^*p < 0.05$ (cited from reference Abe et al. (2007))

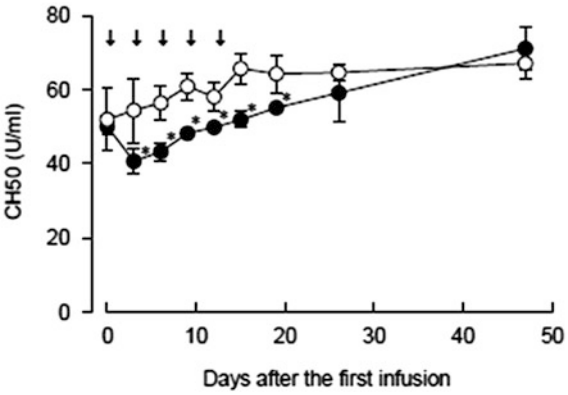


**Fig. 22.2** Effects of repeated infusion of HbV on the rat serum complement. HbV (*closed circles*) or saline (*open circles*) was infused into rats at top-load from the tail vein at the time points indicated by arrows. $N = 3$–5, mean ± SD $^*p < 0.05$ (cited from reference Abe et al. (2007))

Neither HbV nor EV caused the consumption of the complement in rat serum in vitro (data not shown). It is particularly interesting that a repeated-infusion study showed that only first infusion of HbV reduced the complement titer. Despite additional infusions of HbV, gradual recovery of the complement titer occurred, suggesting that additional infusions of HbV did not cause the complement consumption (Fig. 22.2).

Furthermore, multiple administration of EV caused no anaphylactic shock, although ovalbumin-sensitized rats died with symptoms of respiratory distress after the second ovalbumin administration (data not shown). Regarding the evidence collectively, the administration of HbV is apparently safe, without allergic or anaphylactic reactions.

Intravenous injection of liposomes into pigs reportedly induces anaphylactoid reactions at small doses, resulting in circulatory disorder. Therefore, the pig model is regarded as useful for the safety evaluation of liposome drugs. HbV did not cause a significant anaphylactoid reaction in pigs, thereby reconfirming the high biocompatibility of HbV (Sakai H et al. 2012).

### 22.1.6 *Effect of HbV on Immune Response of Rat Splenocytes*

Large amounts of HbV must be transfused to substitute for allogeneic red blood cell transfusion in a clinical setting. Therefore, a considerable number of liposome particles must accumulate in the MPS after HbV infusion, mainly in the spleen and liver (Torchilin 2005). Consequently, it is possible that the immune response fluctuates because of phagocytic cells, which phagocytize HbV, because those cells can become not only positive regulators of immune response as an antigen presenting cells but also negative regulators designated as suppressor macrophages. Reportedly, the production of nitric oxide was involved in its suppressive effect (al-Ramadi et al. 1991; Dasgupta et al. 1999; Schleifer and Mansfield 1993). However, the latter effect has been of little concern, possibly because the amount of liposome used as a drug vehicle is so small that it has no notable negative effect on the immune system in an experimental animal model. This possibility has been addressed recently by our colleagues with the infusion of large numbers of liposomal particles (HbV) (Takahashi et al. 2011). Normal rat splenocytes proliferate well in response to Concanavalin A (Con A) stimulation. However, when the rat splenocytes were taken out 24 h after infusion of HbV (20 % of total blood volume), they failed to proliferate in response to Con A stimulation. When the splenocytes were taken at 7 days after HbV injection, this immune suppression was no longer observed (Fig. 22.3). These results show a transient effect of HbV infusion on immune response. The time course of the suppression appeared to be correlated with that of accumulation and disappearance of HbV from the spleen evaluated based on a histochemical analysis (Sakai et al. 2001; Sou et al. 2005).

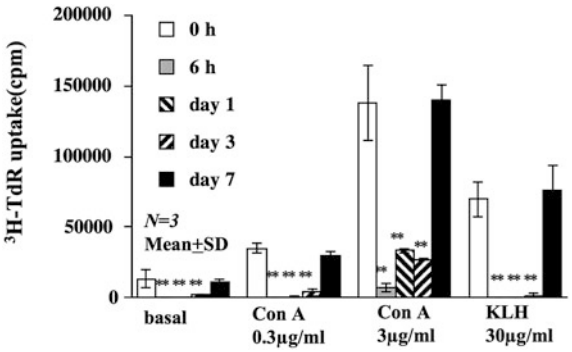


**Fig. 22.3** Effect of the HbV and empty vesicles on proliferation of Con A-stimulated rat splenic T cells. Rats were immunized with KLH. After 7 days, they were injected with HbV. Spleens were excised at 6 h, 1, 3 and 7 days later. Bulk splenocytes were stimulated with Con A or KLH. The proliferative response of splenic T cells to Con A and KLH was inhibited from 6 h to 3 days after injection of HbV compared to control (**: $p < 0.01$). No suppression was observed after 7 days. Data are representative of at least three independent experiments and are expressed as the mean ± SD (cited from reference Takahashi et al. (2011))

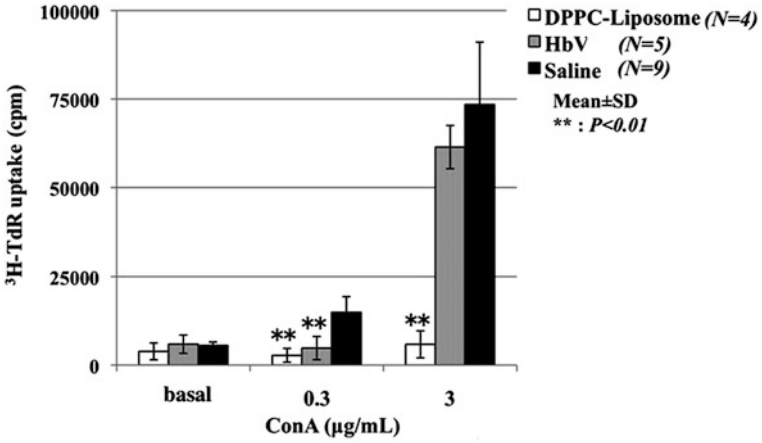


**Fig. 22.4** Effect of DPPC-liposomes on immune suppression. HbV, EV, DPPC-liposome or saline was injected intravenously. The spleen was excised 18 h later. DPPC-liposome induced immune suppression. Data from 2–3 independent experiments are collected and expressed as the mean ± SD (cited from reference Takahashi et al (2011))

The suppressive effect can also be induced by injection of empty liposome particles composed of DPPC only (Fig. 22.4), indicating that transient immune suppression is unavoidable as long as the current liposome particle is used as a vehicle for Hb molecules.

Extensive analyses were performed to elucidate the mechanism underlying this phenomenon. Results obtained so far are the following: (1) T cells were activated and express IL2 receptor (CD25) but were unable to proliferate. (2) T cell proliferation specific to keyhole limpet hemocyanin (KLH) was also inhibited from 6 h to 3 days after the injection of liposomes (Fig. 22.3). (3) Direct cell-to-cell contact was necessary for the suppression. (4) Both iNOS and arginase inhibitors restored T cell proliferation to some degree (Fig. 22.5). (5) Cells that trapped vesicles were responsible for suppression. (6) Most of them expressed CD11b/c, but lacked class II molecules. To summarize these results, the phagocytosis of a large load of liposomal particles by rat CD11b/c$^+$, class II- immature monocytes temporarily renders them highly immunosuppressive. In addition, nitric oxide, possibly produced from cells that phagocytized HbV, is involved in immune suppression. It is noteworthy that the results from an additional experiment showed that HbV infusion did not interfere in the in vivo production of KLH-specific antibody (Fujihara et al.), suggesting that the observed immune suppression is restricted in spleen and not systemic phenomenon.

These data and the effects observed on other blood components revealed the excellent and satisfactory bioavailability of HbV and are expected to guarantee the application of HbV to human as a blood substitute in the near future. Finally, from a different perspective, the observed immunosuppressive effect induced by liposomes might open new fields for the clinical application of liposomes themselves.

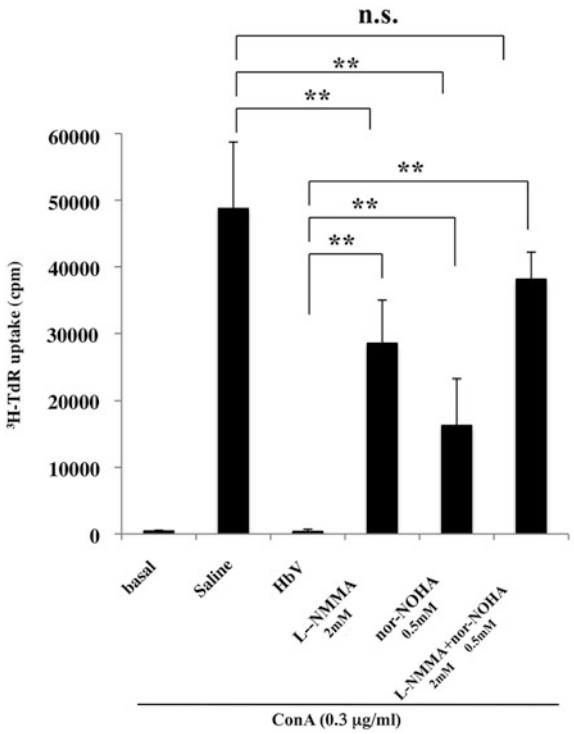


**Fig. 22.5** Effect of L-NMMA and nor-NOHA on suppression of T cell proliferation. Each rat was injected with HbV or saline. Splenocytes were stimulated with Con A (0.3 μg/ml) in the presence or absence of iNOS inhibitor (L-NMMA, 2 mM) or arginase inhibitor (nor-NOHA, 0.5 mM) or both. T cell proliferation was restored in the presence of each inhibitor to a certain degree. Significant inhibition disappeared in the presence of both inhibitors, suggesting that both iNOS and arginase were involved in the suppression. Data from two independent experiments were collected experiments were collected and expressed as the mean ± SD ($N = 5$) (cited from reference Takahashi et al (2011)). **: $p < 0.01$ ns: not significant

## 22.2 Conclusion

In this chapter, we presented the excellent biocompatibility of HbV with human blood cells, human plasma proteins in vitro and rat immune systems in vivo. Along with several lines of evidence, our data demonstrate that HbV are promising candidates for use as artificial oxygen carriers.

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# Chapter 23
# Polymerized Human Placenta Hemoglobin: Organ Protective Effects and Alternative Clinical Uses

**Tao Li, Chengmin Yang, Jin Liu, Jiaxin Liu and Wang Hong**

## 23.1 Introduction

As a life saving intervention, blood transfusions are necessary in emergency and routine clinical practice (Goodnough et al. 1999). But the donated blood for transfusion has many inherent limitations: (1) It has a short stored shelf life and must be used within 42 days; (2) It must be stored in a refrigerated environment; (3) The 2,3-diphosphoglycerate (2,3-DPG) may lose during storage, resulting to increased oxygen ($O_2$) affinity and impaired $O_2$ unloading capacity in tissues; (4) It has a risk of carrying blood borne pathogens such as viral hepatitis and HIV; (5) Blood typing and cross-matching should be performed before transfusion. The incidence of fatal ABO-incompatible transfusions remains the leading cause of deaths resulting from blood transfusion (Chen et al. 2009). These inherent limitations force us to reassess the safety of current blood transfusion. In addition, hospitals and blood banks often experience shortages of donated blood, especially in China. The army also need sufficient blood supply as strategic reserves to improve combat casualty care. Thus, developing a blood substitute is important and urgently required.

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Polymerized human placenta hemoglobin (PolyPHb) is initially developed as blood substitutes by the research group of Professor Chengmin Yang, mainly for patient with hemorrhagic shock and trauma (Li et al. 2006a, b). The procedure of its preparation is highly controlled and conforms to the principles of good manufacturing practice (GMP). Briefly, purified and viral inactivated fresh human placenta hemoglobin was modified with pyridoxal phosphate to achieve optimal $O_2$ affinity. After cross-linkage with glutaraldehyde, PolyPHb was subject to ultrafiltration and molecular sieve chromatography to harvest to final product. Table 23.1 lists the key physico-chemical characteristics of PolyPHb. As a promising hemoglobin-based oxygen carriers (HBOCs), it allows transporting more $O_2$ to hypoxia tissues owing to its higher $O_2$ affinity, lower viscosity and smaller mean diameter than human red blood cells. Based on these merits, we have designed a series studies to exploring its alternative uses from several years ago. In the following section, we will summarize the results of present animal studies about PolyPHb and discuss the potential alternative uses of PolyPHb in clinical situations.

## 23.2 Animal Studies About PolyPHb

In the 2000s, we began exploring the alternative uses of PolyPHb. A series of animal studies were employed, including isolated heart Langendorff model, left anterior descending coronary artery (LAD) ligation model, cardiopulmonary (CPB) model, forced running model, renal I/R injury model and hemorrhagic shock model.

### *23.2.1 PolyPHb for Langendorff-Perfused Rat Heart*

The first report about PolyPHb's cardioprotective effect was published in 2009. In that study, a Langendorff-perfused isolated rat heart model was employed. The heart was arrested with and stored in St. Thomas' solution (STS, an extracellular

**Table 23.1** The key physico-chemical characteristics of PolyPHb

| Parameters | Results or range |
|---|---|
| Placenta hemoglobin (gHb/dL) | 4-6 |
| Tetrameric hemoglobin | <10 % |
| Dimeric hemoglobin | <3 % |
| Methemoglobin (%) | <3 % |
| $P_{50}$* (mm Hg) | 10–24 |
| Colloid Osmotic Pressure (mm Hg) | 10–25 |
| Osmolality (mosm/kg) | 290–320 |
| Molecular Weight (kDa) | 200–240 |
| pH | 7.4 ± 0.2 |

* $P_{50}$: the $PO_2$ level at that hemoglobin is half-saturated with $O_2$

crystalloid cardioplegic solution) with or without PolyPHb (0.5 gHb/dL). The results showed that PolyPHb could improve cardiac functional recovery after 8-h cold storage and 2-h reperfusion (Fig. 23.1). The myocardial infarct size, necrosis and apoptosis were also greatly reduced after PolyPHb treatment (Li et al. 2009a, b). In 2010, by use of Langendorff model, we tested the effect of PolyPHb on cold storage- and warm ischemia-induced injury. PolyPHb with a lower concentration of 0.1 g Hb/dL exhibited similar protective and antiapoptotic effects on isolated rat heart suffering from 8-h cold storage or 40-min warm ischemia and 2-h reperfusion (Fig. 23.2) (Li et al. 2010a, b). Another study in our lab evaluated the influence of deoxygenated PolyPHb pretreatment on Langendorff-perfused heart, and compared with classical cardioprotective method ischemia preconditioning (You et al. 2011). The results demonstrated that there were no significant differences of cardiac functional recovery, enzyme release and histopathological changes between these two groups. Both deoxygenated HBOC pretreatment and ischemia preconditioning greatly improved the cardiac contractile performance recovery and attenuated myocardial enzyme releases.

### *23.2.2 PolyPHb for LAD Ligation-Induced I/R Injury*

In 2011, we pretreated the rat with 0.1gHb/kg PolyPHb to investigate the potential effect of PolyPHb on heart with 30-min LAD occlusion and 2-h reperfusion (Wu et al. 2011). PolyPHb pretreatment improved cardiac functional recovery,

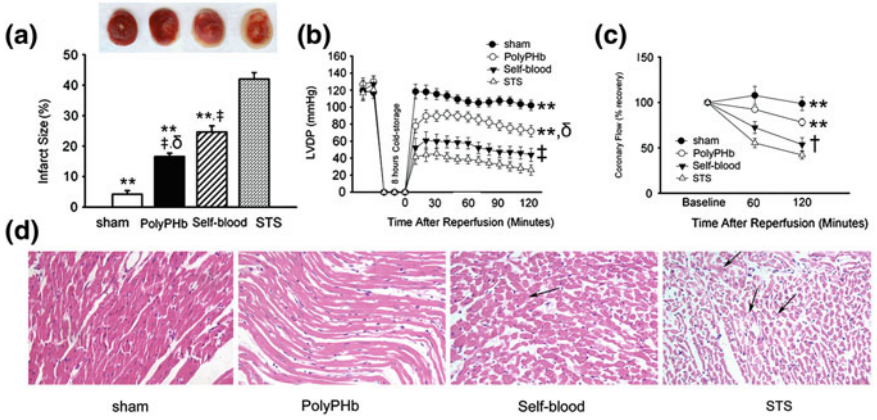


**Fig. 23.1** **a** Representative TTC-stained myocardial sections (n = 5–7, 5–6 slices per heart). **b** LVDP at baseline and during 2-h reperfusion (n = 18–20). **c** Coronary flow rate at baseline and time points of 60 and 120 min of reperfusion (n = 18–20). **d** Representative photomicrographs of HE-stained left ventricular tissue sections. Magnification × 400. *Arrows* indicate the locations of cellular swelling, fatty changes or hyaline changes (n = 5). Values are presented as mean ± SEM. $^{**}P < 0.01$ versus STS group; $\dagger P < 0.05$, $\ddagger P < 0.01$ versus sham group; $\delta P < 0.05$ versus self-blood group

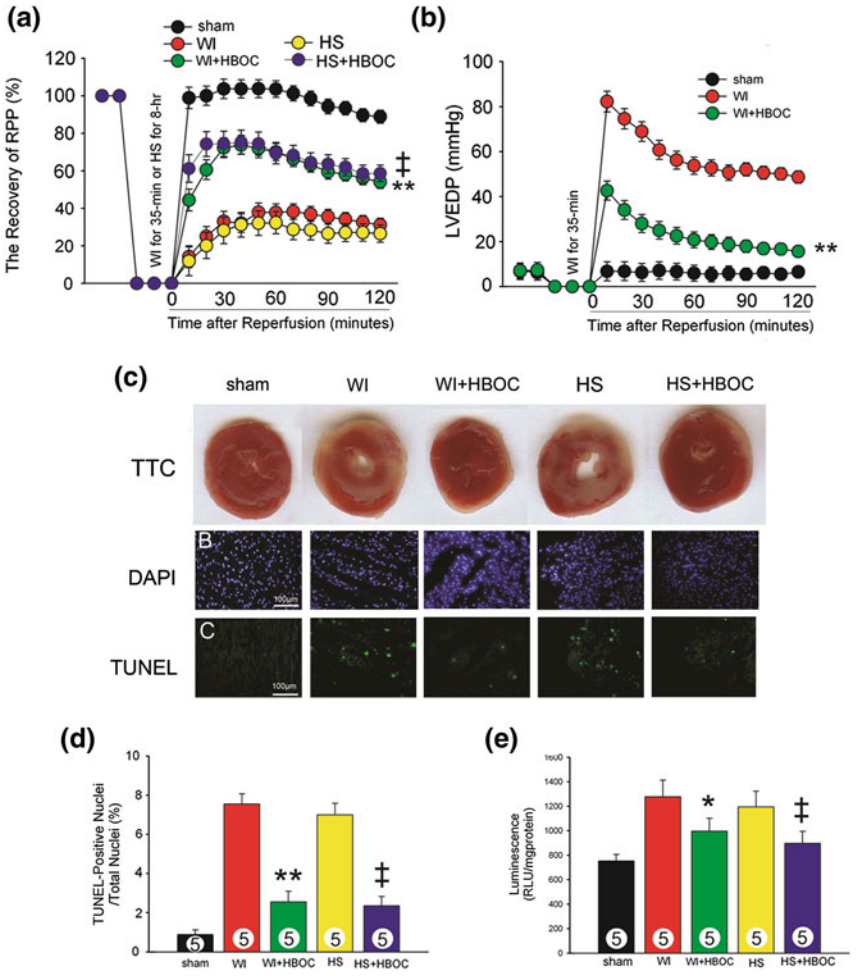


**Fig. 23.2** **a** The recovery of cardiac RPP, the product of LVDP and HR, during reperfusion (n = 13–15). **b** The LVEDP of sham group, WI group and WI + HBOC group during baseline and 2-h reperfusion (n = 13–15). **c** Representative photomicrographs of TTC- and TUNLE-stained hearts. Scale bar: 100 μm (n = 5, 5 fields of each specimen). **d** The percentages of apoptotic cells. **e** Caspase-3 activity assay. Values are expressed as mean ± SEM. Circles inside bars denote n per group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the WI group; ‡$P < 0.01$ versus the HS group

as evidenced by elevated heart rate and systolic/diastolic performance during reperfusion when compared to the control group. The myocardial histopathological changes and enzyme release were also significantly reduced in the PolyPHb group (Fig. 23.3).

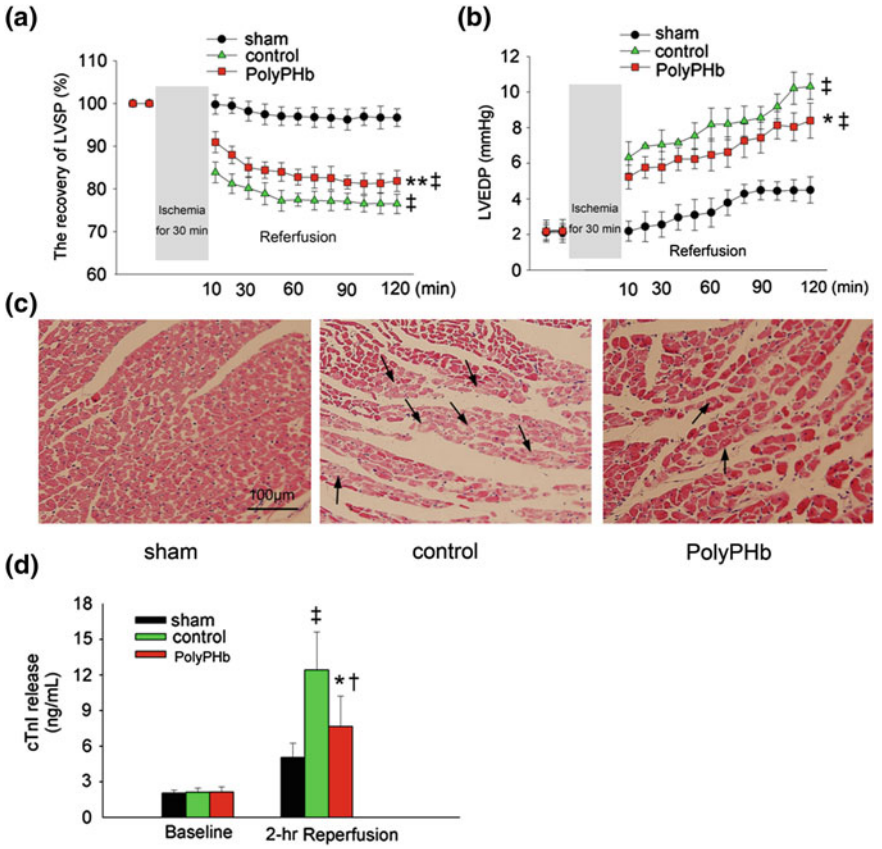


**Fig. 23.3** The recovery of HR (**a**) and LVEDP (**b**) of the 3 group hearts (n = 15). **c** Representative photomicrographs of HE-stained left ventricular tissue sections. Magnification × 400, scale bar: 100 μm. *Arrows* indicate the locations of acute myocardial necrosis, cellular swelling or fatty changes (n = 5). **d** The release of cTnI in the 3 group hearts at 10-min baseline and 2-hr reperfusion (n = 8). Values are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group; †$P < 0.01$ and ‡$P < 0.001$ versus the sham group

### *23.2.3 PolyPHb for Dog Heart During CPB*

Beagle dog CPB model was established in a standard fashion with cannulation of the ascending aorta, superior and inferior vena cava, and left ventricle for venting. STS with or without 0.1 g Hb/dL PolyPHb were infused into the aortic root after aortic clamping to achieve cardiac arrest that was maintained for 2 h, then the aorta was declamped and the heart was reperfused for 2 h. The results showed that the heart rate, cardiac output and cardiac $O_2$ consumption were greatly preserved by PolyPHb. The releases of cardiac enzymes including creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin-I (cTnI) were also greatly reduced.

Moreover, the cellular swelling, fatty changes and hyaline changes were significantly lessened, suggesting that PolyPHb was protective for heart during CPB.

### 23.2.4 PolyPHb for Cardiac Dysfunction Caused by Intense Exercise

Another study about PolyPHb's organ protective effect was performed by use a rat forced running model (Li et al. 2012a). As we know, ultra-endurance exercise causes cardiac damage even in apparently healthy individuals, manifesting as elevated biomarkers of cardiac cell damage in the systemic circulation and a transient reduction in cardiac function. In our study, an obvious cardiac dysfunction was observed after 5-h exercise and the effect of PolyPHb pretreatment (0.1 gHb/kg) was investigated. The results of echocardiography revealed that PolyPHb pretreatment significantly inhibited intense exercise-induced decrease of left ventricular fractional shortening (LVFS) (Fig. 23.4a). In addition, with the increase of cardiac function, the cardiac enzyme releases (CK-MB and cTnI) were remarkably inhibited in comparison to the control group. Therefore, we demonstrated that PolyPHb was effective to protect heart against intense exercise-induced cardiac dysfunction.

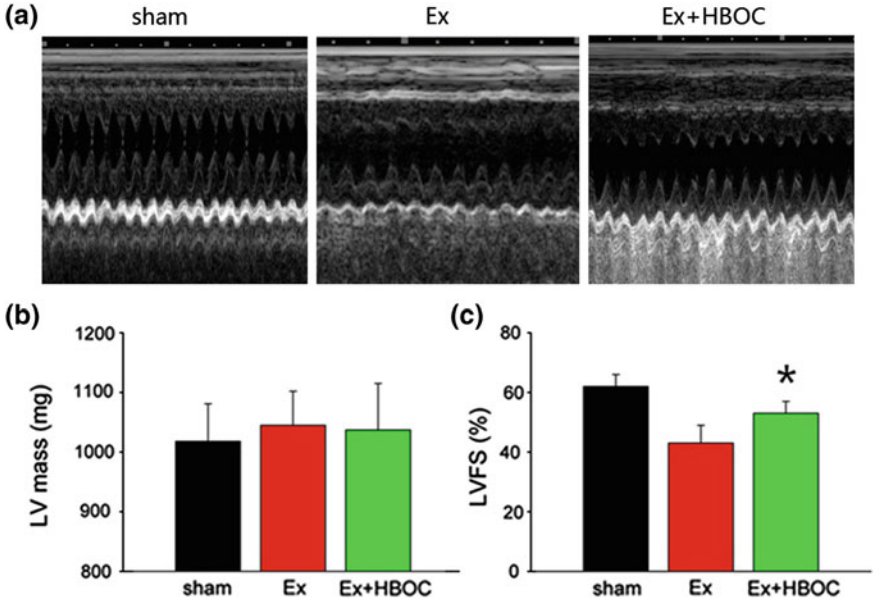


**Fig. 23.4** HBOC attenuated intense exercise induced LV systolic dysfunction. **a** Representative M-Mode echocardiogram in rat LV short axis for each group. **b** Rat LV mass in each group. **c** Rat fractional shortening in LV short axis (LVFS) in each group. Values are expressed as mean ± SEM (n = 5). * $P < 0.05$ versus the Ex group. LV, left ventricular

### 23.2.5 PolyPHb for Renal I/R Injury

In addition to heart, the effects of PolyPHb on other organs were also investigated. We firstly investigated the effect of PolyPHb on renal I/R injury. A renal I/R model was established by 45 min bilateral renal pedicle cross-clamping and 24 h reperfusion. Our study demonstrated that pretreatment with PolyPHb (0.1 gHb/kg) could protect kidney from such I/R injury and inhibited I/R-induced inflammatory response (Fig. 23.5) (Li et al. 2012b). Another important finding of this study is that venous injection of PolyPHb with dosage of 0.1 gHb/kg would not significantly alter the hemodynamic parameters such as blood pressure and heart rate, which might be the basis of its protective effect (Fig. 23.6).

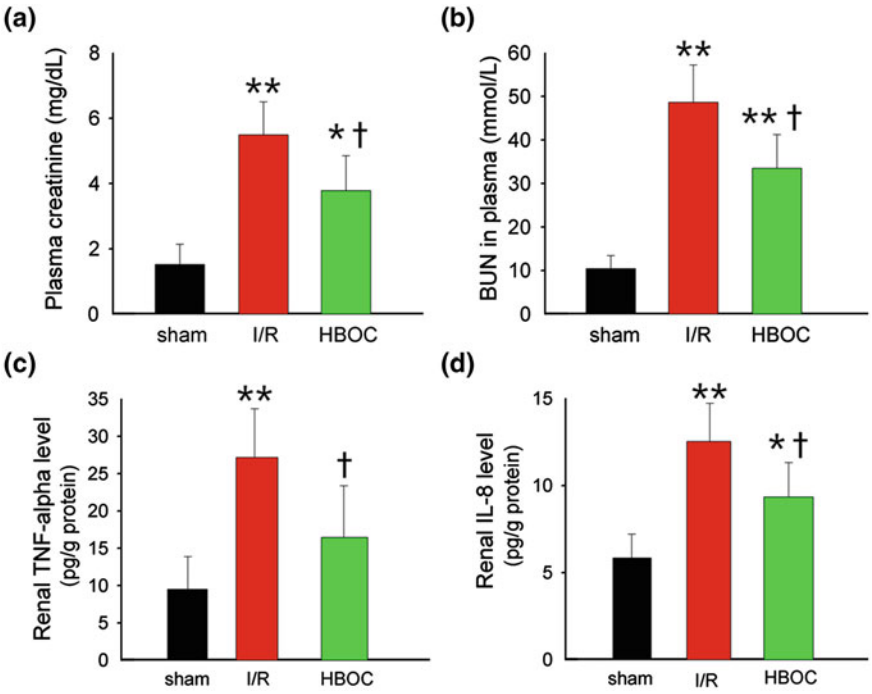


**Fig. 23.5** The releases of creatinine (**a**) and BUN (**b**) in the plasma after renal I/R injury. The levels of TNF-α (**c**) and IL-8 (**d**) in the renal tissue after I/R injury. Values were presented as mean ± SD (n = 15). *$P < 0.05$ and **$P < 0.01$ versus the sham group; †$P < 0.05$ versus the I/R group. *BUN* blood urea nitrogen, *TNF-α* tumor necrosis factor-α, *IL-8* interleukin-8

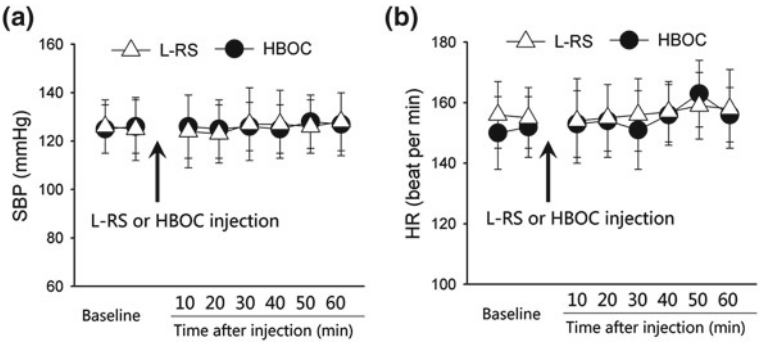


**Fig. 23.6** The SBP (**a**) and HR (**b**) before and after HBOC injection. Values were presented as mean ± SD (n = 15). *HBOC* hemoglobin based oxygen carrier, *HR* heart rate, *L-RS* Lactated Ringer's solution, *SBP* systolic blood pressure

### *23.2.6 PolyPHb for Hemorrhagic Shock-Induced Lung Injury*

For lung injury protection, PolyPHb is also a promising candidate. In an in vivo rat model, we proved that hemorrhagic shock impaired pulmonary function (e.g. reduced $PaO_2/FiO_2$ ratio) and promoted TNF-α and IL-8 release in lung tissue (Li et al. 2013). Resuscitation with PolyPHb not only ameliorated the blood pressure recovery and pulmonary function, but also depressed the elevation of inflammatory cytokines release, indicating a promising protective effect on lung injury (Fig. 23.7).

## 23.3 Mechanisms for Its Protective Effects

As the largest reservoir for $O_2$ and nitric oxide (NO) in the body, hemoglobin acts as an important mediator for oxidative stress and nitroso-redox balance. Thus, most of the mechanic studies on PolyPHb focused on oxidative stress, nitrative stress, and mitochondria, the greatest cellular source of reactive oxygen species (ROS). Firstly, we showed that PolyPHb decreased inducible nitric oxide synthase (iNOS) expression but did not affect eNOS/peNOS expression and nitrite reduction, suggesting that the protective effects of PolyPHb are partially mediated by iNOS inhibition (Li et al. 2009a). After PolyPHb treatment, the oxidative/nitrative injury was also greatly alleviated as evidenced by the increased activity of superoxide dismutase (SOD) and reduced formations of malondialdehyde (MDA), hydrogen peroxide ($H_2O_2$) and peroxynitrite ($ONO\bar{O}$), which is beneficial to restore the nitroso-redox imbalance caused by I/R injury (Li et al. 2009a, 2012a). Besides, our further studies clearly indicated that PolyPHb preserved mitochondrial redox potential ($E_h$), elevated mitochondrial SOD activity and decreased

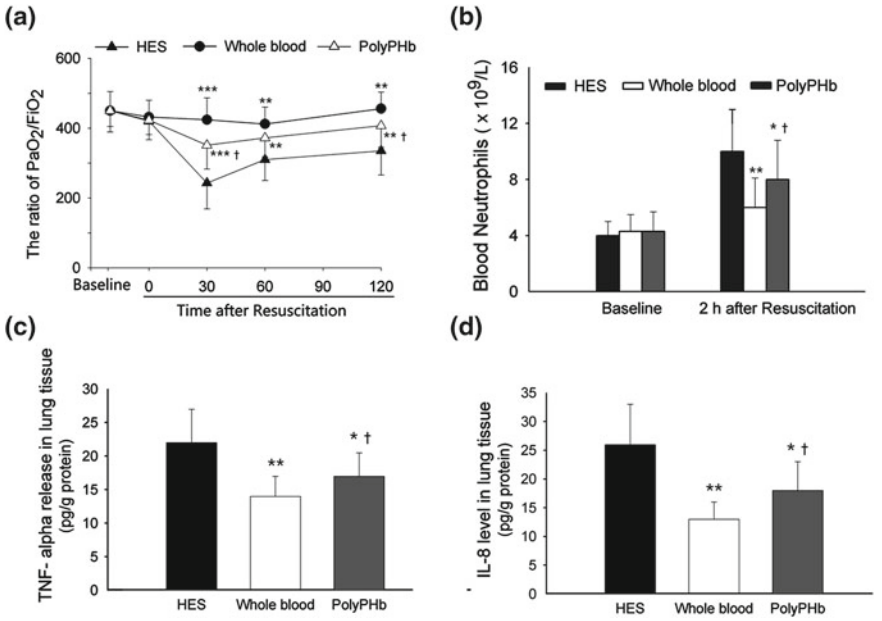


**Fig. 23.7** **a** The PaO2/FiO2 at baseline and during the period of resuscitation. **b** The counts of neutrophils in blood. The levels of TNF-$\alpha$ (**c**) and IL-8 (**d**) in the lung tissue. Values were presented as mean ± SD (n = 15-18). *$P < 0.05$ and **$P < 0.01$ and ***$P < 0.0001$ versus the HES group; †$P < 0.05$ versus the whole blood group. PaO2/FiO2: the ratio of arterial oxygen tension/fraction of inspire oxygen. *IL* interleukin, *TNF* tumor necrosis factor, *WBC* white blood cell

levels of mitochondrial $H_2O_2$ and MDA, indicating that the mitochondrial oxidative damage caused by I/R injury was attenuated. Moreover, the mitochondrial function and ultrastructure were also markedly improved by PolyPHb, further suggesting a prominent role of PolyPHb on cardiac mitochondria. Therefore, the protective effect of PolyPHb might be implicated in restoration of nitroso-redox balance and attenuation of mitochondrial oxidative stress (Fig. 23.8) (Li et al. 2010a). As we mentioned above, PolyPHb treatment can significantly inhibited myocardial apoptosis by increasing Bcl-2/Bax ratio and decreasing iNOS-derived NO production and capase-3 cleavage (Li et al. 2009a). So we believe antiapoptotic effect might be also involved in PolyPHb's protective effects.

## 23.4 Possible Alternative Uses of PolyPHb

Initially, PolyPHb was developed as a blood substitute to treat patients with hemorrhagic shock and trauma (Li et al. 2006a, b). Owing to its optimized $O_2$ affinity, viscosity and size, PolyPHb is able to facilitate microcirculation perfusion

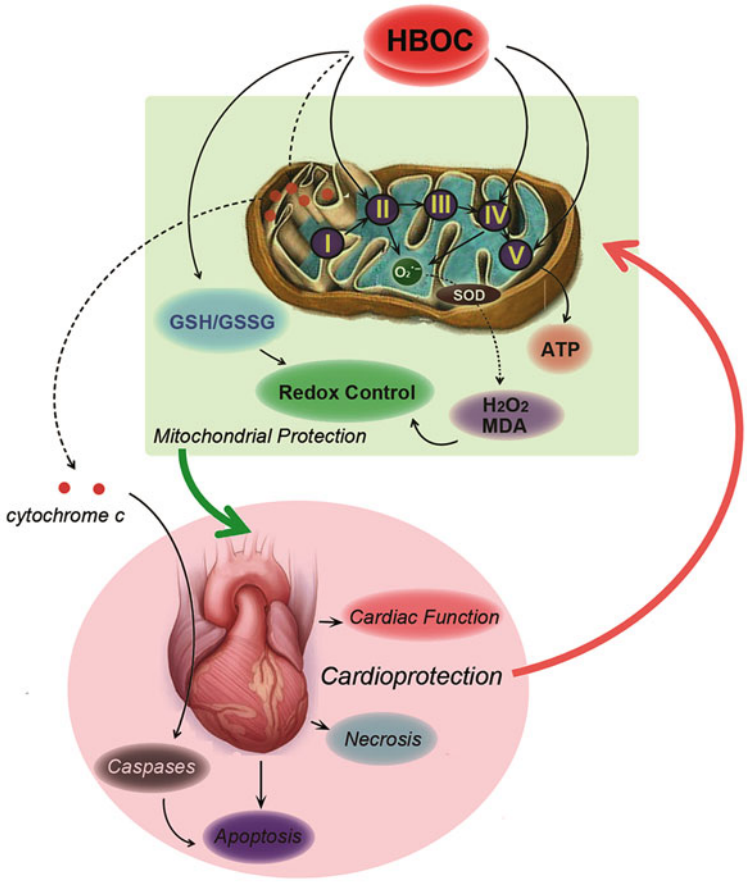


**Fig. 23.8** Proposed mitochondria-myocardium-mitochondria pathway for the cardioprotectie effect of HBOC. I indicates complex I; II indicates complex II (SDH); III indicates complex III; IV indicates complex IV (COX); V indicates complex V (ATP synthase)

and provide sufficient tissue $O_2$ preloading. These advantages make PolyPHb an attracting protective agent for organs or tissues suspected to suffering from I/R injury. I/R injury is a common phenomenon in clinical settings, which usually companied with severe complications, poor prognosis and even death. For example, in cardiac surgery, heart have to be arrested to experience a period of ischemia and then must be reperfused, so I/R injury is unavoidable. Present data of animal studies support our hypothesis that PolyPHb can be used before cardiac surgery to alleviate following I/R injury. As a protective agent, the application of PolyPHb can be expanded to protecting lung, kidney, liver, brain, intestine, and other organs or tissues that may impair by I/R injury. In our opinion, the excellent $O_2$ transporting capacity of PolyPHb makes it also be suitable for elite and

recreational athletes in endurance exercise, like marathon and iron-man triathlon, as well as travelers who will climb high mountains or enter the plateau.

However, the prerequisite for these alternative applications is that the product is safe and stable. According to some recently published clinical trials, there are several complications associated with systemic administration of HBOCs, such as coronary and cerebral vasospasm, gastrointestinal side effects, chest and abdominal pain (Chen et al. 2009; Jahr et al. 2002; Winslow 2003; Natanson et al. 2008). In our previous studies, we did not observed any of these complications, probably for that the dosage used in these studies (0.1 gHb/dL) was lower than that reported in the clinical trials. A large dose of PolyPHb (1-3 gHb/dL) would also bring a rapid increase in blood pressure, even though it returned to baseline within 10 min. We believe that PolyPHb is not perfect as most other HBOC products, a lot of works are still required to improve its quality.

## 23.5 Summary

Based on the results of animal studies, some alternative applications of PolyPHb have been revealed, including in perioperative organ protection and the field of sports medicine. We believe that these studies will in turn facilitate and promote the development of more safe and effective HBOC products.

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# Chapter 24
# Low Volume Resuscitation with HBOCs in Hemorrhagic Shock

**P. S. Reynolds, R. W. Barbee and K. R. Ward**

## 24.1 Introduction

Restricted fluid resuscitation is the new norm for victims of hemorrhage following trauma. In the recent past, the goal of fluid resuscitation was restoration of blood pressure to normotensive levels, and the best way to achieve that goal was thought to be by aggressive infusion of crystalloids. It was reasoned that normalization of blood pressure was equivalent to restoration of adequate organ perfusion, and would therefore prevent post-shock complications such as multiple organ failure and death. However, hemodilution, increased bleeding, hemodynamic decompensation, lung damage secondary to late inflammatory responses, and increased mortality were common sequelae following unrestricted crystalloid resuscitation (Coimbra et al. 2007). By contrast, current thinking emphasises restoration of tissue perfusion with relatively small or restricted volumes of resuscitation fluid, with substitution of standard crystalloids or blood with hypertonic or hyperosmotic fluids. Because these are essentially plasma expanders with limited oxygen carrying capacity, it has been suggested that augmentation with hemoglobin [Hb]-based oxygen-carrying compounds (HBOCs) would improve oxygen delivery to the tissues (Kramer et al. 2004).

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## 24.2 Hemorrhagic Shock and Resuscitation

Shock is a state of hypoperfusion at the cellular level that occurs when the delivery of oxygen ($DO_2$) to the tissues falls below the oxygen uptake ($VO_2$) required to sustain aerobic metabolism. Global oxygen delivery $DO_2$ is the product of cardiac output CO and arterial blood oxygen content $ct_aO_2$. The mismatch or imbalance between tissue supply and demand that occurs during hemorrhage results from reductions in the circulating blood volume and blood flow, which in turn leads to reduced CO and $ct_aO_2$. In the early compensatory phase of hemorrhagic shock, tissue oxygen demand can be met by an increase in oxygen extraction from the arterial blood; this can be monitored by progressive reduction in the mixed venous oxygen saturation ($S_vO_2$) and an increase in the arterio-venous oxygen gradient, which is the difference in oxygen content between the arterial and venous blood ($C_aO_2$–$C_vO_2$). During this phase, mean arterial pressure MAP and oxygen consumption $VO_2$ are maintained at roughly constant levels (Fig. 24.1). However, as $DO_2$ declines with increasing blood loss and compensatory mechanisms begin to fail, oxygen extraction becomes insufficient to meet cellular demands. As a result, the difference between oxygen supply from $VO_2$ and tissue oxygen demand begins to increase. The oxygen supply:demand mismatch at any given time is the *oxygen deficit.* When a critical tipping point in $DO_2$ is reached (*critical oxygen delivery* $DO_{2,crit}$), oxygen supply to the tissues becomes delivery-dependent, and microvascular responses (reduced microvascular resistance and increased capillary recruitment) to progressive hypoxia become inadequate to meet tissue oxygen demand. As a result, the mitochondria can no longer support aerobic metabolism,

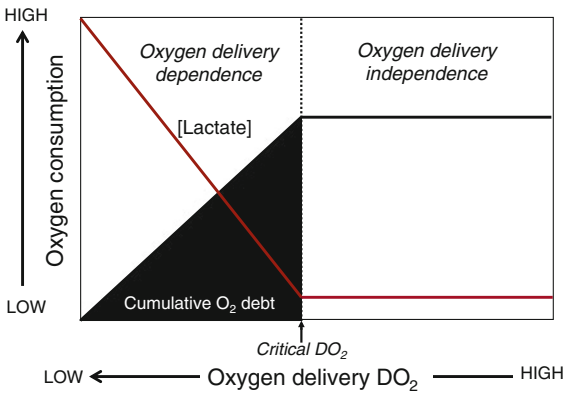


**Fig. 24.1** Schematic of the relationship between oxygen consumption $VO_2$ and oxygen delivery $DO_2$. As $DO_2$ to the tissues declines, $VO_2$ demands are met by an increase in cardiac output and oxygen extraction, and a decrease in mixed venous oxygen saturation $SvO_2$ (*delivery-independence*). Below critical $DO_2$, compensatory oxygen extraction mechanisms fail, and $VO_2$ becomes dependent on $DO_2$ (*delivery-dependence*). Increase in lactate may occur as tissue hypoxia increases. Thus the relationship between $VO_2$ and $DO_2$ is approximately biphasic

and cell metabolism begins to transition to anaerobic pathways. On the systemic level, both MAP and $VO_2$ decline abruptly, and lactate increases (Fig. 24.1). Thus, physiological responses to the decline in $DO_2$ that accompanies progressive hemorrhage are roughly biphasic (Walley 1996). However, there is considerable regional variability in tissue resistance to hypoxia. For example, whereas skeletal muscle may tolerate hypoperfusion-induced cell damage for upwards of several hours, other tissues such as the gut and brain may sustain severe damage after only a few minutes, and before significant decline in conventional vital signs such as MAP can occur. More prolonged cellular hypoxia and cell damage result from activation of inflammatory cascades and free radical production. Hypoperfusion and tissue hypoxia are also the primary initiators of trauma-induced coagulopathy (TIC; Brohi et al. 2003). In other words, the shock process is initiated by blood loss and hypovolemia, but the inflammatory cascades triggered by prolonged hypoperfusion exacerbate the shock state, and result in eventual tissue damage and organ failure (Angele et al. 2008). Thus adequate shock resuscitation must account for the time-dependent summation of oxygen deficits (*oxygen debt*) resulting from hypoperfusion injury that accrues from point of injury to its resolution (Fig. 24.2). The clinical importance of oxygen debt and its prompt and adequate resolution cannot be overstated; oxygen debt has been shown to be the best quantitative predictor of mortality and morbidity in both experimental and clinical settings (Rixen and Siegel 2005; Barbee et al. 2010).

Pragmatically, hemostasis and restoration of intravascular volume are the immediate needs for severely bleeding patients. However correction of hypoperfusion will gain in importance over the longer term (hours to days) to avoid

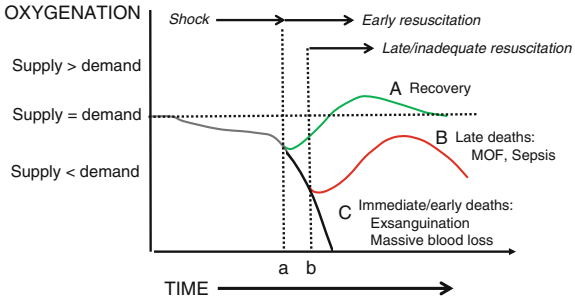


**Fig. 24.2** Schematic of the relationship between oxygen deficit and time to irreversible hypoperfusion injury. The difference between oxygen supply and demand to the tissues (oxygen deficit) increases during shock; the cumulative difference is the oxygen debt. (*A*) Recovery occurs with repayment of oxygen debt by adequate and timely resuscitation to correct hypovolemia and hypoperfusion, and prevent initiation of cell-damage-mediated inflammatory cascades. (*B*) Inadequate and/or delayed resuscitation may restore volume deficits but too late to reverse cellular damage caused by prolonged hypoperfusion; late death from organ damage and mulitple organ failure may result (*B*). Severe on-going hemorrhage and hypovolemia result in immediate or early death (*C*)

irreversible cell damage, multiple organ failure, and late death. Animal studies indicate that there is an increased probability of irreversible cell damage and death if oxygen debt is allowed to accumulate past a certain time-debt threshold (Siegel et al. 2003, Rixen and Siegel 2005). For example, in a canine model of delayed and partial volume resuscitation with colloids, animals given either no resuscitation fluids or <30 % of their shed blood volume within 2 h after hemorrhage continued to accumulate oxygen debt even though no further blood loss was incurred. These animals subsequently developed severe and highly lethal cell damage, even though they were fully resuscitated after the 2 h delay. In contrast, animals receiving at least 75 % shed blood volume within 2 h all survived with minimal or no cell damage, and no organ failure (Siegel et al. 2003). Thus, assessment of a given fluid resusictation strategy as "adequate" involves both appropriate volumes of fluid and appropriate timing of the intervention to correct hypoperfusion injury.

Unfortunately there are numerous logistic problems inherent to measuring oxygen debt (Barbee et al. 2010), not least of which is location of patient care (for example, $S_vO_2$ is difficult to monitor outside an ICU setting). As a result, a number of clinical surrogate indicators of shock have been proposed for monitoring purposes. These include hemodynamic indices (such as MAP <90 mmHg), low central venous oxygen saturation $S_{cv}O_2$, and markers of metabolic acidosis such as lactate >2 mM or base deficit <−6 mM (Rixen and Siegel 2005). None of these measures is satisfactory, as none can fully assess the degree of oxygen debt accumulated from the point of injury over the continuum of treatment. Thus the challenge common to all resuscitation strategies following a shock event is the timely restoration of intravascular volume and correction of tissue hypoxia in the frequent absence of obvious and easily measured clinical markers of shock status.

## 24.3 Low Volume Fluid Resuscitation Defined

### *24.3.1 Strategies and Working Definitions*

The major fluid resuscitation strategies outlined in this review are summarised in Table 24.1. In the civilian setting, standard Prehospital and Advanced Trauma Life Support (PHTLS and ATLS, respectively) shock resuscitation protocols for severe hypovolemia include IV fluid resuscitation with crystalloid (usually Lactated Ringer's or normal saline). Because crystalloids shift fairly rapidly out of the intravascular space into the interstitital space, PHTLS and ATLS guidelines recommend resuscitation volumes of approximately three times the shed blood volume (the 3:1 rule). Patients with on-going signs and symptoms of hemorrhagic shock, acute hemorrhage, hemodynamic instability and/or inadequate oxygen delivery are candidates for blood transfusion to correct these problems (Napolitano et al. 2009). Until recently, resuscitation goals were fairly aggressive; fluids were

**Table 24.1** Summary of standard of care (PHTLS, ATLS) and low-volume fluid resuscitation strategies. See text for details

| Strategy | Therapeutic goals | Therapeutic endpoints | Recommended fluids | Theoretical advantages |
|---|---|---|---|---|
| *PHTLS, ATLS* | Volume replacement; maintain SBP at levels sufficient to maintain end-organ perfusion; restore and maintain normal vital signs | SBP 80–90 mm Hg<br>Palpable radial pulse<br>Increased mentation<br>Maintenance of normal vital signs | Crystalloids 3 × SBV<br>FFP:PRBC | Rapid volume replacement and early restoration of perfusion |
| *Permissive hypotension* | Maintain MAP at levels sufficient to maintain end-organ perfusion; "early" correction of coagulopathy; avoidance of exsanguination | MAP 70 mm Hg<br>SBP 90 mm Hg | 1:1 FFP:PRBC | Reduction of fluid and blood product requirements<br>Maintenance of "clean" operative field |
| *Delayed resuscitation* | No or minimal fluids until surgical control of hemorrhage | N/A | N/A | No clot disruption<br>No rebleeding |
| *Volume-controlled resuscitation* | Expansion of intravascular fluid volume | MAP 70 mm Hg<br>SBP 90 mm Hg<br>CO↑<br>$DO_2$↑ | Hyperosmotic crystalloids<br>Hyperoncotic colloids<br>Combination | Minimization of logistic footprint<br>(Weight,space)<br>Reduction in fluid and blood product requirements<br>Reductions in fluid overloading and edema<br>Improved CO, $DO_2$ |

administered until vital signs returned to 'normal', that is systolic blood pressure >100 mm Hg and pulse <100 bpm.

In contrast to conventional fluid resuscitation protocols, the therapeutic goal for low volume fluid resuscitation (LVFR) is to enable survival with the minimum of fluid so that the injured patient can reach definitive care (such as surgical hemorrhage control and blood transfusion), while avoiding or minimising the problems of hemodilution and secondary bleeding associated with large-volume infusions of crystalloids. However, there is considerable ambiguity in the working definitions of "restricted" or "low volume" resuscitation strategies, the therapeutic target endpoints, and resuscitation goals to be achieved. To date, there are three variations of the LVFR strategy: permissive hypotension, delayed resuscitation, and volume-controlled resuscitation (Kreimeier et al. 2000).

#### 24.3.1.1 Permissive Hypotension

The stated therapeutic goal of permissive hypotension is to maintain blood pressure at levels sufficient to maintain end-organ perfusion, while avoiding exsanguination and achieving early correction of coagulopathy (Duchesne et al. 2010; Curry and Davis 2012). This mode of resuscitation has been widely adopted by the United States military for management of combat casualties, especially during the first echelons of care when casualties are transported to combat support hospitals. Because of the problems inherent to obtaining blood pressure readings in the field, pulse quality and mental status are used to assess perfusion "adequacy"; altered mental status and weak or absent radial pulse are assumed to represent shock (Salomone and Pons 2007). For intra-operative applications, the goal of permissive hypotension is to decrease blood loss, both to maintain a "clean" operative field and reduce blood transfusion requirements (Kreimeier et al. 2000). Permissive hypotension is central to the concept of damage control resuscitation (DCR); this strategy emphasizes maintenance of systolic blood pressure (SBP) at approximately 90 mmHg, or mean arterial pressure (MAP) of approximately 70 mmHg (near the autoregulatory limit; Cowley 1992). Recommended resuscitation fluids for permissive hypotension strategies involve transfusion of 1:1 fresh frozen plasma: packed red blood cells (FFP:PRBC), and no or limited crystalloid administration. Limited evidence for benefit of permissive hypotension dates back over 50 years from studies of surgery patients (Eckenhoff and Rich 1966), and more recently, from civilian (Dutton et al. 2002; Morrison et al. 2011) and combat trauma (Holcomb 2003) patient populations. It is generally assumed that fluid and blood product requirements will be much less compared to standard normotensive resuscitation strategies (e.g. Morrison et al. 2011)

#### 24.3.1.2 Delayed Resuscitation

Delayed resuscitation is the strategy of limiting fluid therapy by eliminating or reducing the otherwise routine administration of pre-hospital and/or ED fluids (usually crystalloids) until surgical control of hemorrhage has been achieved. Thus, there are no specific fluid administration recommendations. The therapeutic goal to be achieved is minimisation of total blood loss by avoidance of clot disruption that may result from excessive fluid administration before surgical hemorrhage control. Limited evidence (Kwan et al. 2003) exists for two civilian field trials of trauma patients with either predominantly penetrating (Bickell et al. 1994) or blunt (Turner et al. 2000) injury.

#### 24.3.1.3 Volume Controlled Resuscitation

Small-volume, or volume-controlled, resuscitation emphasizes improved "efficiency" of fluid administration by adjusting fluid composition to allow "physiological equivalence" to conventional crystalloids, but at volumes much smaller than those required by crystalloid infusion (Kramer et al. 2004). The definition of physiological equivalence has not been formally specified, but may be considered to refer to expansion of intravascular fluid volume (Holcomb 2003; Kramer 2003). This strategy has been advocated primarily for military or tactical applications, where small volume requirements provide considerable logistic advantages, such as minimizing weight carried by medics, and allowing conservation of scarce fluid resources in austere or dangerous environments (Pearce and Lyons 1999; Dubick and Atkins 2003; Holcomb 2003). Small volume fluid resuscitation has also been evaluated for intra-operative use in cardiac surgery (Kreimeier and Messmer 2002; Azoubel et al. 2008)

Recommended fluids for volume controlled resuscitation are hyperosmotic crystalloids (e.g. hypertonic saline, HTS, 7.2–7.5 % NaCl), hyperoncotic colloids (e.g. hydroxyethyl starch HES, albumin), or a combination of fluids, e.g. hypertonic saline with dextran [HSD] (Kreimeier and Messmer 2002). Tactical Combat Casualty Care volume guidelines (Salomone and Pons 2007) suggest administration of HES (Hespan®, Hextend®) up to two boluses of 500 mL each; this represents a fluid load of approximately 7–14 mL/kg for a 70 kg subject. More extreme goals have been proposed with the objective of reducing fluid loads to 4 mL/kg (the approximate equivalent of a 250 mL bag of fluid for a 70 kg subject) before definitive resuscitation (Kreimeier and Messmer 2002; Rocha-e-Silva and de Figueiredo 2005). Justification for 4 mL/kg loading volume was originally based on preliminary data from a dog model of severe hemorrhagic shock (base excess −8.5 meq/L); 4 mL/kg was apparently the minimum volume of a hyperosmotic NaCl solution allowing survival of 10/10 test animals for >6 h (Velasco et al. 1980). Nearly 90 comparative trials have been performed to evaluate low-dose (250 mL or 4 mL/kg) infusions of HTS, HSD, and/or isotonic crystalloid on a variety of patient populations (Kramer et al. 2004), including cardiac surgery

patients (Azoubel et al. 2008). Limited evidence suggests some benefit in terms of reductions in fluid overloading and edema, and improved CO (Kreimeier and Messmer 2002; Azoubel et al. 2008).

### *24.3.2 Current Practice and Problems*

There are no recognized current guidelines for LVFR strategies or for supplementary use of HBOC in the civilian setting. If the immediate therapeutic goal of LVFR is restoration of intravascular volume to a level sufficient to ensure autoregulation of the critical organs (brain, heart, and lungs) without inducing rebleeding through mechanical disruption of blood clots, then the required target MAP will be at least 60–80 mmHg. However, MAP is a notoriously poor indicator of end organ perfusion (Ward et al. 2001). Volume-based guidelines are similarly imprecise. Current military/Tactical Field Care recommendations (Salomone and Pons 2007) suggest a 500 mL bolus of Hextend to restore or maintain field vital signs (palpable radial pulse, improved mentation) until definitive care; the bolus is repeated once if no improvement is noted in 30 min. This represents an initial fluid load of approximately 6.3–6.7 mL/kg, or a total of approximately 12–14 mL/kg over 30 min for a 75–80 kg patient. However these volumes may be grossly inadequate for correcting cell and organ damage related to persistent (>2 h) hypoperfusion. Using data derived from the previously described pre-clinical oxygen debt model (Siegel et al. 2003), we estimated minimum volume requirements for a colloid to be 18–20 mL/kg, approximately double the Tactical Field Care recommended maximum. We could not find equivalent data for hyperosmotic solutions (Barbee et al. 2010)

At present, the impact of LVFR on mortality and morbidity cannot be determined reliably from human clinical trials. There is some evidence of efficacy for all three LVFR strategies; however evidence is compromised by poor study quality. Nearly all studies performed to date have the potential for considerable bias because of inadequacies in randomisation and allocation concealment and studies that are too small and under-powered.

Specific problems with permissive hypotension relate to practical difficulties in achieving a target MAP because of patient auto-resuscitation, and confusion as to the actual therapeutic goal, namely whether the true target is an a priori defined MAP (essentially a population measure), or hypotension which is relative to the individual patient baseline and usually unknown at point of injury. Randomised controlled trials evaluating efficacy of permissive hypotension are sparse. One trial compared maintenance of target systolic blood pressures of 100 mmHg (control) and 70 mmHg in hypotensive (SBP <90 mmHg) trauma patients, but found no difference in survival to hospital discharge (Dutton et al. 2002). However fluid types and volumes infused were not reported, and numerous other study limitations indicate the potential for significant bias (Alsawadi 2012). A field trial comparing hypotensive (SBP 70 mmHg) vs standard fluid resuscitation (SBP 110 mmHg) is

currently enrolling patients; however this trial is not utilising HBOCs, and of this date of writing no data are available (*ClinicalTrials.gov* Identifier NCT01411852). Interim results from a second on-going trial of permissive hypotension without HBOCs (Morrison et al. 2011) comparing hypotensive trauma patients maintained at target MAP of 65 mmHg (HMAP) versus MAP of 50 mmHg (LMAP) suggest a potentially higher risk of early death in the HMAP group (8/46 vs. 1/44), primarily because of exsanguination and coagulopathy. However SBP of both groups was considerably lower than that defined by DCR recommendations, averaging approximately 75 mmHg, and observed MAPs were similar for the two groups in spite of different specifications for target MAP. Contrary to expectation, total fluids did not differ statistically between groups, although the HMAP group received larger volumes of blood products: 3.3 (95 % CI 1.9–3.9) L vs. 1.6 (95 % CI 0.9–2.3) L.

Small-volume fluid resuscitation with either hyperosmotic crystalloids or colloids may have benefit in terms of decreased fluid loading and minimisation of blood transfusions (Kreimeier and Messmer 2002; Azoubel et al. 2008), but there seems to be little or no benefit in reducing mortality, and a possible increased probability of harm. Our re-analysis of previously-published 30-day mortality data for trauma and hemorrhage patients in a meta-analysis of resuscitation trials utilising hypertonic crystalloids (Wade et al. 1997) showed no evidence of benefit (odds ratio 0.894; 95 % CI 0.726–1.101; $p = 0.291$).[1] The largest randomised controlled clinical trial to date for out of hospital administration of low-volume hypertonic fluids showed no difference in 28 day survival between groups receiving hypertonic solutions and these receiving standard of care normal saline; rather there was evidence of increased mortality in HTS groups (Bulger et al. 2011). A recent systematic review of HES resuscitation fluids (Hartog et al. 2011) concluded that use of HES is not associated with significant fluid savings in comparison to crystalloids when fluid administration was directed to achieve a specific hemodynamic target, although the effects of low volume administration on outcome were not specifically addressed. There are significant safety concerns associated with the use of high molecular weight HES, including coagulopathy, anaphylactoid reactions, nephrotoxicity, acute renal failure and increased mortality (Hartog and Reinhart 2009; Zarychanski et al. 2013). Approval of newer formulations of HES (130/0.4) were based on equivalence studies in the context of acute hypovolemia during elective surgery; however studies were designed too poorly to address issues of safety and efficacy (Hartog et al. 2011; Reinhart and Takala 2011).

Delayed resuscitation is essentially a logistic, rather than a clinical, strategy; fluid resuscitation is "restricted" by eliminating the prehospital phase of fluid administration. However a significant disadvantage of delayed resuscitation (and indeed a problem common to all three restricted-volume resuscitation strategies) is the risk of under-resuscitation during the early stages of injury. As previously

[1] Comprehensive meta-analysis [computer programme]. http://www.meta-analysis.com

stated, early reversal of hypoperfusion and oxygen debt within a fairly restricted time window (possibly <2 h) is essential to avoid acute traumatic coagulopathy, tissue and organ damage, organ failure, and late death. However, at present there is no adequate technology or unambiguous constellation of signs and symptoms to allow emergency personnel to identify occult bleeding, or if patients without overt signs and symptoms of shock are already critically volume-depleted (Stern 2001; Stern et al. 2001).

## 24.4 The Role of HBOCs in Low Volume Fluid Resuscitation

### *24.4.1 Rationale/Theoretical Advantages*

Although resuscitation with non-blood fluids can restore intravascular volume and increase CO, it does not necessarily improve oxygen delivery to the tissues. In addition, large amounts of crystalloids can result in a number of deleterious iatrogenic effects, such as reduced oxygen delivery, acidosis, hyper-inflammatory responses, lung injury and ARDS, dilutional coagulopathy, oedema, and compartment syndromes (Kaplan et al. 1999; Coimbra et al. 2007). Therefore it is thought that addition of an oxygen-carrying adjunct to small-volume intravascular volume expanders should serve to improve or maintain oxygen delivery until hemorrhage control is achieved and/or blood is available, and without the problems associated with large volume crystalloid infusions. Some early generation HBOCs had volume expansion properties (DCLHb, HBOC-301), possibly resulting from high oncotic pressures and increased plasma protein; however the "volume sparing" effects may have also been an artifact of severe vasoconstriction induced by NO scavenging (Kramer et al. 2004)

### *24.4.2 Evidence*

No oxygen carriers are currently approved for clinical use in the United States. As a result, there are no standard doses or formulations of HBOC or HBOC cocktails for low-volume resuscitation/hemorrhagic shock indications, and no accessible information on unit costs. Although several Phase II and III trials examining HBOC feasibility for trauma and elective surgery have been completed (www.ClinicalTrials.gov), there are few published data available and many previously evaluated products are no longer available

A systematic search of MEDLINE, CINHAHL, TRIP, *ClinicalTrials.gov*, Cochrane reviews, and a hand-search of the published literature was conducted in April June 2012. Search terms used in the databases are given in Appendix 1. A meta-analysis of clinical trials using HBOCs has been published previously

(Natanson et al. 2008), although only 4 of the 16 trials analysed specifically referred to resuscitation volumes of 250 mL or less. Our searches did not turn up additional HBOC human trials with published data, and were subsequently restricted to animal models of hemorrhage resuscitation (Appendix 2). Trials were included if a specified HBOC product was compared to a conventional resuscitation fluid (blood, crystalloid, colloid) at infusion volumes less than the equivalent shed blood volume, or if hypotensive and/or delayed resuscitation was specifically mentioned, and if clinically relevant outcomes (mortality, hemodynamics, oxygenation) were reported. Duplicated, isodilution/anemia, and bypass studies were excluded. Trials were summarised using RevMan5.2.[2] Trial quality was assessed in terms of risk of bias in five bias domains (Higgins et al. 2011), and for measures of quality such as sample size, number of groups, and sample size per group. Physiological outcomes were evaluated in terms of mortality (proportions), improvement of at least 50 % from the end of shock in hemodynamic support (MAP > 60 mm Hg, CO increase, HR decrease), and improvement of at least 50 % in oxygenation ($SvO_2$, $VO_2$, $DO_2$, and/or tissue oxygenation) within 2 h of administration.

#### 24.4.2.1 Animal Models and Study Quality

We identified 24 animal trials that met inclusion criteria (Appendix 2). Trials originated from a limited number of research groups; 13/24 (54 %) of included studies were from only three groups of collaborators. Animal species used were swine ($n = 14$), dogs ($n = 6$), rats ($n = 3$), and sheep ($n = 1$)

Overall, study quality was poor, and extracted studies were too heterogeneous to attempt a formal meta-analysis that would provide meaningful results. Risk of bias was high (Fig. 24.3). Only 3 trials mentioned that animals were randomly allocated to treatment, only one described the method of randomisation used, and only one indicated that treatment order was randomised. Only 2 studies reported blinding of any outcome assessment. Selective reporting of multiple outcomes was common, and large numbers of multiple pair-wise comparisons, significance testing on large numbers of outcome variables, and failure to account for time dependencies contributed to bias towards false positives. Failure to control for animal body size within trials contributed substantial variation to estimates relying on weight-based metrics (e.g. total blood volume or hemorrhage volume in ml/kg); coefficients of variation for animal body mass (when reported) ranged from 3 % to over 100 % (median 25 %). The variety of anaesthetic protocols added another element of confounding because of potential effects on baseline hemodynamics and physiological responses to hemorrhage. Primary outcomes were clearly identified in only 2 trials.

[2] Review Manager (RevMan) [Computer program]. Version 5.2. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012.

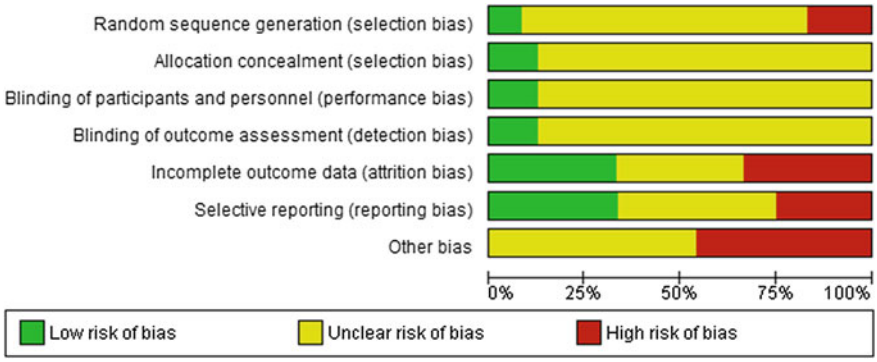


**Fig. 24.3** Risk of bias summary of risk judgements in five bias domains presented as percentages across animal-based studies of restricted volume resuscitation with HBOCs ($n = 24$). "Low" risk: methods are described in sufficient detail to allow adequate assessment; "Unclear risk": there is insufficient information to allow adequate assessment; "High risk": study groups were clearly dissimilar before intervention, outcome measures/assessments are likely to be influenced by deficiencies; and/or analyses are likely to contribute to false positives. Summary graph was generated in RevMan5.2 (The Cochrane Collaboration, 2012). [For more details, see Higgins et al. (2011) and Cochrane Collaboration, Appendix D: *Cochrane risk of bias tool*]

#### 24.4.2.2 Hemorrhage Models

Hemorrhage models used were extremely diverse; 11 were "pressure" models (e.g. animals were hemorrhaged to a pre-specified MAP), 6 were volume-hemorrhage models (animals were hemorrhaged by removing a pre-specified percentage of the estimated total circulating blood volume), 4 trials used both volume and pressure criteria (animals were hemorrhaged to a pre-specified percent volume without allowing MAP to drop below a predetermined threshold), and the remainder were uncontrolled solid organ injury models (liver laceration/crush, $n = 2$), or a two-phase controlled/uncontrolled hemorrhage ($n = 1$), using pre-determined time points or predetermined blood volume as targets. Trials varied considerably in size; median total sample size was 24 (range 9–116), with the numbers of groups tested ranging between 2 and 8 with a median group size of 6.

Median hemorrhage volume was approximately 30 mL/kg (range 16–45 mL/kg), median MAP at the end of hemorrhage was 31 mmHg (range 20–55 mmHg), and animals were maintained in a "shock state" at a specified hypotensive target prior to resuscitation for anywhere between 5 and 60 min. Shock severity could be assessed independently of MAP for 21 studies, when post-hemorrhage clinically-relevant shock markers (such as lactate or base deficit) were reported. Reported lactate levels at the end of the shock period ranged from <2 mM (indicating relatively mild shock with minimal or no oxygen debt) through moderate (3.5–5 mM) to high (>8 mM, an indication of severe shock); median lactate was 3.8 mM.

**Table 24.2** HBOC products used in 24 reviewed studies; study references are given in Appendix 2

| Hemoglobin type | Trade name | Alternate name | Manufacturer |
|---|---|---|---|
| Diaspirin cross-linked hemoglobin | HemAssist | DCLHb | Baxter International Corp., Deerfield IL |
| Bovine hemoglobin glutamer-200 | Oxyglobin | HBOC-301 | OPK Biotech LLC, (formerly Biopure Corp)., Cambridge MA |
| Bovine hemoglobin glutamer-250 | Hemopure | HBOC-201 | OPK Biotech LLC., Cambridge MA |
| Zero-linked bovine polymerised hemoglobin | OxyVita | Zero-link Hb | OxyVita Inc., New Windsor NY |
| Polymerised stroma-free human hemoglobin | PolyHeme | Poly SFH-P | Northfield Laboratories Inc., Evanston IL |
| PEG conjugated human hemoglobin | Hemospan | MP4; MP4OX | Sangart Inc., San Diego CA |

#### 24.4.2.3 Resuscitation

The HBOC test solutions covered in this review are summarised in Table 24.2. The animal trials included here tested HBOC-201 (Hemopure®, OPK Biotech; 11 studies), or HBOC-301 (Oxyglobin®, OPK Biotech; 7 studies); One or two trials each tested zero-link Hb (OxyVita™, OXYVITA, Inc.), diaspirin crosslinked hemoglobin (DCLHb/HemAssist®, Baxter Corp.), Poly SFH-P (PolyHeme®, Northfield Labs, Inc.), or MP4OX (Hemospan®, Sangart Inc.) solution.

Resuscitation protocols were likewise extremely heterogeneous. Six studies included a "no resuscitation" group as one of the controls. Comparison solutions included autologous shed blood, Hextend® or another HES (Hespan®, pentastarch), and/or crystalloid (lactated Ringer's, Ringer's acetate, normal saline, 7.5 % hypertonic saline). Median resuscitation volumes for HBOC solutions and HBOC cocktails (consisting of an HBOC plus crystalloid and/or colloid carrier solutions) were approximately 10 mL/kg, ranging from 3 to 81 mL/kg, resulting in resuscitation volumes that were roughly 30 % of estimated hemorrhage volume. At least 8 studies infused fluid to maintain a prespecified MAP target (e.g. 60 mmHg), and 12 studies provided additional fluid support (crystalloid, blood) to "maintain hemodynamics" after the initial fluid intervention. Duration of monitoring to terminal endpoint ranged from 2 h to 5–7 days.

#### 24.4.2.4 Physiological Outcomes

Mortality was low in the context of the time frame designated for each study; 17 studies had no mortality in at least one study arm, and 11 studies had no mortality or only 1 isolated death. The utility of HBOC solutions for providing MAP support was difficult to determine because of the practice of fluid supplementation and/or repeated bolusing to maintain a pre-designated MAP target. For those studies with

no reported target MAP, MAP was increased to at least 60 mmHg within 2 h in 17 studies, with 15 studies reporting at least equivalent performance of HBOC solutions relative to controls. Of 19 studies reporting CO, HBOC solutions were equivalent or superior to controls in 16 studies, and performed less well in 3 studies. Effects were relatively short-lived in most cases (<4h), possibly because of greater vasoreactivity and/or higher systemic vascular resistance induced by the infusions, although estimates of effect duration are confounded because of the relatively short monitoring periods (generally $\leq$ 4 h) of many studies. Ten studies reported equivalent or better improvement of global oxygen delivery $DO_2$ by HBOC infusion relative to controls; however interpretation is confounded when $VO_2$ and $DO_2$ were estimated from CO using the Fick equation, instead of independent determination from respiratory gas monitoring.

Fourteen studies reported equivalent or better resolution of metabolic acidosis (in terms of lactate and/or base deficit) compared to controls, and three were worse, two studies reported no resolution with any resuscitation fluid, and three studies were indeterminate (lactate or base deficit values were not sufficiently altered by the hemorrhage/shock insult to allow resolution with resuscitation). Studies measuring tissue oxygenation reported that HBOCs performed at least as well as controls, although at least one study showed that the effects were relatively transient.

#### 24.4.2.5 Physiological Responses: Summary

Results are equivocal as to efficacy of HBOC augmentation of LVFR solutions. MAP and tissue oxygenation support appear to be at least as good in the short term as support provided by conventional resuscitation fluids. It is possible that HBOC solutions act to facilitate oxygen transfer to the tissues at the microcirculatory level, rather than global oxygen delivery *per se* (Driessen et al. 2003), which may in part explain why few studies demonstrated overall superiority of HBOC solutions in terms of global oxygen transport or delivery. If HBOC solutions can target microcirculatory oxygen transfer, this may be because of the ability of unencapsulated hemoglobin to traverse the microcirculation through the plasma space (thought to be the major site of resistance to oxygen transport; Pittman 2005). Regardless of mechanism, if HBOC solutions do improve perfusion relative to control solutions, then it can be predicted that there should be a concomitant lessening of cell and end-organ damage. Few published rationales for use of HBOCs as an oxygen therapeutic adjunct have explicitly considered oxygen debt repayment as a therapeutic goal (but see Siegel et al. 1997); to our knowledge, none has done so for low-volume formulations. However, several swine studies have examined the potential of low-volume HBOC-201 infusions for minimising cell damage and histopathological changes (Sampson et al. 2003; York et al. 2003; Fitzpatrick et al. 2005; Johnson et al. 2006; Moon-Massat et al. 2012). In these studies, better survival and hemodynamic support were reported for animals receiving HBOC, but there was either no reduction, or an increase, in markers of end-organ damage. However, it is difficult to unambiguously assess these effects

relative to control solutions. The majority of these studies reported additional experimental interventions (e.g. additional blood and crystalloid, multiple or repeat bolusing to maintain blood pressure, etc.). Relatively few studies examined the use of low-volume HBOC in the context of uncontrolled hemorrhage, where presumably LVFR strategies would realize their greatest potential benefit.

In general, short-term mortality rates and pre-resuscitation lactate levels were low. This suggests that study animals, although critically volume depleted, were usually not at high risk of critical hypoperfusion and immediate substantial oxygen debt. Several animal studies (e.g. Jones et al. 1969; Rixen et al. 2001; Siegel et al. 2003) have demonstrated wide variation in both hemorrhage volume loss and/or MAP for any given oxygen debt, primarily because of individual variability in compensatory capability. As a result, animals having the same weight-specific (mL/kg) loss of blood over a given period of time (and therefore the same perceived insult) can have widely varying and clinically significant differences in oxygen debt, and thus widely-varying degrees of clinically relevant shock injury. Consequently the potential of LVFR and HBOCs for correcting hypoperfusion injury or reducing mortality and morbidity cannot be assessed reliably at present.

## 24.5 Adverse Effects

Use of early generation HBOCs was associated with serious adverse events, including myocardial infarction, vasoconstriction, systemic hypertension, and nephrotoxicity, with little evidence of survival benefit (Natanson et al. 2008). Underlying mechanisms of injury may have related to nitric oxide scavenging, production of reactive oxygen species, effects on autonomic regulation (Jahr et al. 2002; Bernard et al. 2011) and high methemoglobin levels contributing to coagulopathy (Moallempour et al. 2009). Other potentially deleterious effects of HBOCs include cross-reactivity reactions (Hamilton and Kickler 2007), and coagulopathic effects (Jahr et al. 2002; Jahr et al. 2008) Optical interference with laboratory measurements may confound interpretation (Jahr et al. 2005).

Adverse effects might be minimised if HBOC solutions are regarded as adjuncts rather than volume replacement fluids, and infused at very low concentrations and/or volumes (Jahr et al. 2002). To date, many clinical trials have administered HBOC products at levels equal to 100 % shed blood volume for prolonged periods. However, manufacturer's recommended doses are generally much lower. For example, veterinary applications of HBOC-301 (Oxyglobin®) recommend a one-time loading dose of 30 mL/kg IV at a rate not to exceed 10 mL/kg/h; recommended doses are usually 10–15 mL/kg IV for dogs and 3–5 mL/kg IV for cats (Kudnig and Mama 2002). Other HBOCs, such as zero-link Hb(OxyVita™), have demonstrated minimal effects on coagulation at recommended doses of 2–3 mL/kg (Jahr et al. 2008). *Ex vivo* trials suggest laboratory measurements of lactate (a clinically relevant shock marker) are not affected by HBOC concentration *per se*, but there may be unexpected synergisms, as considerable measurement error

seems to occur at higher (>5 mM) lactate levels in the presence of HBOC (Jahr et al. 2005). Newer compounds such as zero-link Hb (OxyVita$^{TM}$) and MP4OX are characterised by high molecular weights, lower hemoglobin concentrations (from 10–13 g/dL to 4–6 g/dL), and oxygen dissociation curves left-shifted from $P_{50}$ of 30–40 mmHg to 6 mmHg (Jahr et al. 2002).

High molecular weight formulations, especially at low doses, are expected to lessen the extent of extravasation (with subsequent vasoconstriction and hypertensive overshoot) typical of early products (Pittman 2005; Cabrales et al. 2009). However the low $P_{50}$ of these products will inherently limit the amount of product that can be given, as such low values may perpetuate tissue hypoxia. Animal studies have indicated that there are continuing concerns with vasoactivity, acute hypertensive effects, and pulmonary, renal, and hepatic damage (Johnson et al. 2006), although further purification and removal of the low molecular-weight Hb component promises to mitigate some of these effects (Rice et al. 2008).

Apart from these concerns, the long-term effects of limited or hypotensive resuscitation strategies themselves on mortality and morbidity are still unknown. This is especially pertinent for certain patient groups, such as those with pre-existing hypertension (where patients would be expected to have a higher pressure threshold for harm), or elderly patients. Likewise, there are few data to support or refute claims of efficacy in the presence of polytrauma, especially blunt injury, although there is evidence that prolonged hypotension in the presence of traumatic brain injury (TBI) is highly detrimental (Chestnut 1997; Cooper et al. 2004). There are no guidelines for MAP thresholds with respect to nature of the injury, patient tolerance of a "target" MAP, duration of tolerance, or how tolerance of a predetermined threshold may vary with the resuscitation fluid type, time from point of injury, or rate of resuscitation.

## 24.6 Conclusions

HBOCs as oxygen therapeutic adjuncts in a low volume fluid cocktail of hyperoncotic-hyperosmotic fluid may meet the immediate requirements of volume expansion while conserving blood components, reducing rebleeding, and improving hemodynamics and tissue oxygenation, thus reducing risk of hypoperfusion-related injury. However adverse effects related to dose-related cell damage may offset any benefit that may be present from increased oxygen-carrying capacity or delivery. To date, data from preclinical studies are too sparse and variable in quality to allow generalizations as to efficacy of low-dose HBOC cocktails for trauma resuscitation. It is unlikely that data from orthopedic or cardiac surgery trials will sufficiently inform how HBOCs contribute to tissue oxygenation during LVFR of trauma patients, as very few patients in surgery trials would be either severely hypovolemic or at high risk of significant oxygen debt. More evidence is needed to assess benefit and harm of HBOC in low-dose formulation and LVFR strategies in general, as well as determining optimal

dose/volumes, time of administration, and cost-effectiveness. To determine the efficacy and safety of both LVFR and adjunct HBOCs in the context of traumatic hemorrhage, there is an imperative need for adequately-powered, standardised, high quality animal trials and clinical randomized controlled trials with clearly defined therapeutic goals.

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## A.1 Appendix 1 Literature Search Strategy

Search terms included:

(hemorrhag* OR hemorrhag* OR bleeding OR hypovolem* OR hypovolaem*) AND ((low volume) OR (hypotensive) OR (permissive hypotension)) AND (fluid OR crystalloid OR colloid OR saline OR hypotonic OR hypertonic OR plasma) AND ((HBOC) OR (oxygen carrier))

((low volume) OR (small volume)) AND fluid AND resuscitation AND ((hboc) OR (hemoglobin-based oxygen carrier) OR (oxygen-carrying blood substitute solutions) OR (ocbs)). ((low volume) OR (Small volume)) AND fluid AND resuscitation AND ((HBOC) OR (hemoglobin-based oxygen carrier) OR (oxygen-carrying blood substitute solutions) OR (OCBSS))

Limits: Humans, Animals, Clinical Trial, Meta-Analysis, Randomized Controlled Trial, Review, Clinical Trial, Phase I, Clinical Trial, Phase II, Clinical Trial, Phase III, Clinical Trial, Phase IV, Comparative Study, Controlled Clinical Trial, Journal Article, Multicenter Study, Retracted Publication, Retraction of Publication, Technical Report, Validation Studies

An internet search was conducted in April and May 2012 using *Grey matters: A search tool for evidence-based medicine* (Canadian Agency for Drugs and Technologies in Health [CADTH], 2012), using the search terms HBOC or (hemoglobin) OR (hemoglobin)

A search of *ClinicalTrials.gov* using the search term (HBOC) or (oxygen carrier) resulted in 7 relevant trials, of which 2 were suspended, 1 terminated, 4 completed and one recruiting. No data were available in published form.

## A.2 Appendix 2 Animal Studies Included in Review

1. Bosman RJ, Minten J, Lu HR, Van Aken H, Flameng W. (1992) Free polymerized hemoglobin versus hydroxyethyl starch in resuscitation of hypovolemic dogs. Anesth. Analg. 75: 811–817.

2. Buehler PW, Mehendale S, Wang H, Xie J, Ma L, Trimble CE, Hsia CJC, Gulati A (2000) Resuscitative effects of polynitroxylated αα-cross-linked hemoglobin following severe hemorrhage in the rat. Free Radical Biology & Medicine 29: 764–774.
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# Chapter 25
# Ischemic Rescue with Hemoglobin-Based Oxygen Carriers

**Raymond C. Koehler**

**Abbreviations**

| | |
|---|---|
| 20-HETE | 20-hydroxyeicosatetraenoic acid |
| CBF | Cerebral blood flow |
| CO | Carbon monoxide |
| Hb | Hemoglobin |
| HBOC | Hemoglobin-based oxygen carrier |
| MCA | Middle cerebral artery |
| MCAO | Middle cerebral artery occlusion |
| NO | Nitric oxide |
| $PO_2$ | Partial pressure of oxygen |
| $P_{50}$ | $PO_2$ at 50 % oxyhemoglobin saturation |
| PEG | Polyethylene glycol |
| RBC | Red blood cell |
| ZL-HbBv | Zero-link bovine Hb polymer |

## 25.1 Introduction

Infusion of chemically modified, cell-free hemoglobin (Hb) has been studied in a variety of experimental pathophysiological models. Most work has been performed in animal models of hemorrhagic shock in which organs become hypoperfused as a result of low perfusion pressure, sympathetic stimulation, and humoral factors that selectively constrict arterioles in specific organs. Ischemia, which can be defined as a reduction in blood flow sufficient to impair oxidative metabolism, can occur in specific organs depending on the severity of hemorrhage

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and the balance between intrinsic autoregulatory vasodilator mechanisms in tissue and vascular sensitivity to sympathetic activation and circulating hormones, such as catecholamines, angiotensin, and vasopressin. In this setting, infusion of a resuscitation fluid that has the ability to carry $O_2$ has the potential to be superior to non-$O_2$ carrying solutions that expand blood volume but dilute the red blood cells (RBCs). Thus, hemoglobin-based $O_2$ carriers (HBOCs) have been effective in models of hemorrhagic shock.

In addition to hemorrhagic shock, which affects perfusion throughout the body, other conditions in which ischemia is restricted to specific tissue may also benefit from cell-free Hb transfusion. In this chapter, the use of cell-free Hb solutions under conditions of arterial occlusion will be reviewed. Much experimental work has been published in models of stroke, but some work has also been performed with ischemia in heart and other organs. The general mechanisms by which acellular Hb may be beneficial in these conditions will be discussed first.

## 25.2 Mechanisms of Improved Oxygenation by HBOCs During Ischemia

The ability of an HBOC to improve tissue oxygenation during occlusion of an artery requires delivery of the HBOC to the ischemic microcirculation through collateral vessels supplied by a non-occluded artery or directly through the occluded artery if occlusion from stenosis or thrombus is incomplete. The potential mechanisms by which an HBOC can improve tissue oxygenation can be divided into those that improve convective transport of $O_2$ into the microcirculation and those that improve the diffusion of $O_2$ from the microcirculation into the tissue.

### *25.2.1 Convective $O_2$ Transport*

Convective $O_2$ transport is generally well regulated to match $O_2$ consumption of each organ, although neurohumoral influences may limit $O_2$ transport to less vital organs under conditions of severe stress. In brain and heart, blood flow is reciprocally related to arterial $O_2$ content such that the product of blood flow and $O_2$ content, which is equivalent to bulk convective $O_2$ transport, is fairly well maintained under conditions of decreased arterial partial pressure of $O_2$ ($PO_2$), decreased hematocrit, or a combination of the two. This relationship is not perturbed when arterial $O_2$ content is boosted by an HBOC under conditions of decreased arterial $PO_2$ and hematocrit (Ulatowski et al. 1998). Thus, arteriolar tone is adjusted during hypoxic hypoxia and anemia to permit blood flow to maintain convective $O_2$ transport. However, this relationship is perturbed when the local perfusion pressure falls because of impaired cardiac function or because upstream resistance is increased by arterial occlusion. Under conditions of partial or

complete arterial occlusion, downstream arterioles will maximally decrease vascular tone. The residual blood flow then becomes passively dependent on arterial blood pressure, the vascular resistance through the partially occluded artery and through small collateral vessels supplied by adjacent arteries, and the downstream vascular resistance in the microcirculation. Vascular resistance depends on both vascular geometry and blood viscosity. Because downstream arterioles in the microcirculation maximally decrease vascular tone during ischemia, one strategy to decrease vascular resistance is to decrease blood viscosity. Consequently, many studies of HBOCs have used large exchange transfusion to decrease hematocrit and blood viscosity while partially offsetting the decrease in RBC $O_2$ carrying capacity by increasing plasma $O_2$ carrying capacity. With early studies in models of complete middle cerebral artery (MCA) occlusion, this approach could increase blood flow in the ischemic region through collateral vessels, particularly if the exchange transfusion was hypervolemic to also increase arterial blood pressure (Cole et al. 1992, 1994). The limitation of this approach in clinical practice is that implementation of an exchange transfusion of 30–50 % of blood volume would require rigorous monitoring and a highly trained staff.

Another potential approach to improve convective $O_2$ transport during complete occlusion of an artery is to augment the dilation of collateral arteries that supply the ischemic region. In the case of cerebral cortex, much of the collateral blood flow between the distribution areas of the three main cerebral arteries arises from small anastomotic arteries on the pial surface rather than through connections among microcirculatory units served by arterioles that penetrate into the cortical tissue. Pial arteries in an ischemic region usually dilate despite the reduction in transmural distending pressure that arises from upstream occlusion. However, the dilation is not always sustained, possibly because of time-dependent changes in release of vasodilatory metabolites such as nitric oxide (NO) and because of increased release of the potent constrictor endothelin (Cao et al. 2009; Yamada et al. 2000). Thus, pharmacological strategies to augment dilation of collateral arteries are one approach for improving delivery of RBCs and an HBOC into the ischemic region.

Although collateral blood flow is rarely sufficient to restore blood flow to normal in a region where the main arterial supply is completely occluded, it could be sufficient to delay the full loss of energy metabolism and cell death until full reperfusion is established by clot removal or dissolution. Boosting convective $O_2$ transport through collateral arteries by augmenting $O_2$ carrying capacity and/or decreasing blood viscosity by transfusion of an HBOC might delay the gradual loss of high-energy phosphates in the ischemic region, particularly if combined with therapy to augment dilation of collateral arteries. In this regard, it is important to ensure that the HBOC itself does not counter vasodilation, for example, by scavenging NO or increasing endothelin release. Another consideration is that chronic hypertension causes inward remodeling of arteries, and the ability to increase collateral blood flow in patients with chronic hypertension may be compromised. Moreover, ischemic risk factors, such as hypertension, hyperlipidemia, diabetes, and cigarette smoking, act to decrease endothelial production of NO (Atochin et al. 2007). NO scavenging by HBOCs may cause additional loss of

NO bioavailability and further limit collateral blood flow. Thus, the ability of an HBOC to improve convective $O_2$ transport is dependent on many factors that could vary within a clinical population.

### 25.2.2 *Diffusional $O_2$ Transport*

Diffusion of $O_2$ from the microcirculation to parenchymal cells depends on spatial $PO_2$ gradients, which, in turn, depend on the geometry of the microcirculation, distances from blood vessels to the various parenchymal cells, the rate of $O_2$ consumption of the different cell types, the distribution of $O_2$ flux through microcirculatory vessels, the diffusivity of $O_2$ in plasma and tissue, and the Hb $P_{50}$ ($PO_2$ at 50 % oxyhemoglobin saturation). Plasma-based HBOCs have the potential to influence the latter three aspects (Tsoukias et al. 2007).

The flux of RBCs through individual capillaries can become relatively heterogeneous during ischemia because the perfusion pressure distal to the occlusion is markedly reduced and because blood viscosity increases at low shear rates. At low perfusion pressures in the feeding arterioles during ischemia, RBCs may preferentially enter a subset of capillaries at the expense of others. Consequently, some capillaries have very low numbers of RBCs entering them, and those that do enter them can have low velocities. On the other hand, all capillaries tend to maintain some flow of plasma at low perfusion pressures. Thus, having an $O_2$ carrier in the plasma will act to increase the relative homogeneity of $O_2$ flux among capillaries (Vogel et al. 1997) and thereby limit pockets of tissue with complete anoxia.

Ordinarily, the flux of $O_2$ across the endothelium depends on the $PO_2$ gradient between blood and tissue, the capillary surface area, and the microvascular hematocrit. RBCs move through a capillary in a single file, and the plasma in the intervening space has a low $O_2$ carrying capacity. Thus, at any instant in time, the surface area for direct diffusion of $O_2$ from the RBC to the endothelium is much smaller than the total endothelial surface area. By having an HBOC that increases the $O_2$ carrying capacity of the plasma, surface area for $O_2$ diffusion at any instant is increased (Vadapalli et al. 2002).

Traditionally, the $PO_2$ gradient from RBCs to endothelium has been thought to be small. However, this gradient can be significant because the low solubility of $O_2$ in plasma offers an effective resistance to diffusion. In support of this concept, transient fluctuations in intra-capillary $PO_2$ have been described at a single recording site as each RBC passes through the capillary. These transients have been described in mesenteric vessels and indicate that $PO_2$ differs between RBC and plasma (Golub and Pittman 2005). In brain olfactory bulb, which consumes relatively high amounts of $O_2$ and has high capillary density and short distances to parenchymal glomeruli cells, the $PO_2$ gradient between RBCs and the intervening plasma may be as much as 30 mm Hg, whereas the gradient from the plasma midway between RBCs and tissue is much smaller (Lecoq et al. 2011). Thus, the

plasma compartment represents a considerable resistance to overall diffusion of $O_2$ from the RBC into tissue.

In this regard, the concentration of $O_2$ in plasma can be markedly increased by the presence of an HBOC. Because of the rapid on- and off-kinetics of $O_2$ on an HBOC and its high mobility in plasma, HBOCs are capable of shuttling $O_2$ from the RBC membrane to the endothelial membrane. Such facilitation of $O_2$ transport from the RBC is expected to increase tissue $PO_2$ (Tsoukias et al. 2007; Vadapalli et al. 2002). In addition to facilitating $O_2$ transport from the RBC, the HBOC itself can provide a net release of $O_2$ as it passes through the microcirculation, depending on its $P_{50}$ relative to tissue $PO_2$ (Kavdia et al. 2002). In essence, having an $O_2$ carrier in the plasma increases the effective surface area for $O_2$ diffusion from RBC and plasma sources.

Interestingly, in non-ischemic brain, exchange transfusion with an HBOC produces constriction that is blocked by a synthesis inhibitor of 20-hydroxyeicosatetraenoic acid (20-HETE) and not by an inhibitor of NO synthase (Qin et al. 2006). Because the synthesis of 20-HETE is $O_2$-dependent and is known to constrict cerebral arteries under other conditions of increased oxygenation (Ohata et al. 2010), vasoconstriction to HBOC transfusion may be viewed as a regulatory response to limit over-oxygenation. During cerebral ischemia, in contrast, exchange transfusion with the HBOC does not cause constriction, and inhibition of 20-HETE synthesis has no effect (Cao et al. 2009). Presumably, the severity of tissue hypoxia reduces 20-HETE synthesis, whereas release of other mediators promotes vasodilation during ischemia.

It has been assumed that the optimal $P_{50}$ for HBOCs should be similar to that of RBC-based Hb. This assumption may hold when the HBOC is replacing much of the RBC mass and the HBOC is to be used to unload most of its $O_2$. However, there may be situations when an HBOC with a low $P_{50}$ is effective. For example, in ischemic conditions with low tissue $PO_2$, an HBOC with a low $P_{50}$ may still unload considerable $O_2$. If the particular HBOC retains the Bohr effect, the acidosis associated with ischemia will increase the $P_{50}$ in vivo and additional $O_2$ will be unloaded. Moreover, when considering that an HBOC can facilitate $O_2$ transport from the RBC to the endothelium, such facilitation may be optimal with a $P_{50}$ that is intermediate between the $P_{50}$ of the RBC-based Hb and the tissue $PO_2$ (Koehler et al. 2008; Mito et al. 2009). Furthermore, some $O_2$ is already unloading from the RBCs when passing through arterioles (Vovenko et al. 2003). Having an HBOC with a low $P_{50}$ would be expected to unload less of its $O_2$ until it reaches capillaries with lower $PO_2$ (Vadapalli et al. 2002). Thus, an HBOC with a low $P_{50}$ may limit precapillary $O_2$ losses and deliver more of its $O_2$ selectively in ischemic tissue with low $PO_2$. Although some of the $O_2$ unloaded in arterioles may reach parenchymal cell mitochondria, some diffuses into venules that cross arterioles (Lecoq et al. 2011). Such a diffusional shunt of $O_2$ results in venular $PO_2$ exceeding tissue $PO_2$. HBOCs with low $P_{50}$ may minimize this loss of $O_2$ by diffusional shunt.

As summarized in Table 25.1, multiple mechanisms are theoretically available for HBOCs to improve tissue oxygenation under ischemic conditions. The relative importance of each mechanism awaits more detailed experimental validation.

**Table 25.1** Potential mechanisms whereby HBOCs can increase $O_2$ delivery to ischemic tissue

| |
|---|
| *Increase convective $O_2$ transport to the microcirculation* |
| • Boost whole-blood $O_2$ carrying capacity by increasing plasma $O_2$ carrying capacity |
| • Decrease hematocrit and blood viscosity and thereby increase collateral blood flow while sustaining whole-blood $O_2$ carrying capacity |
| • Promote dilation of collateral arteries by releasing CO or NO or by removing superoxide |
| *Increase diffusion of $O_2$ from the microcirculation to parenchymal cells* |
| • Deliver more $O_2$ to the capillaries that have a disproportionately low RBC flux and thereby improve the homogeneity of $O_2$ flux among capillaries |
| • Increase the effective capillary surface area for $O_2$ diffusion by filling capillary spaces between RBCs with an $O_2$ source |
| • Facilitate $O_2$ transport from RBCs to endothelium and diminish the $PO_2$ gradient between RBCs and intervening plasma |
| • HBOCs with high $O_2$ affinity may limit pre-capillary $O_2$ loss, selectively unload $O_2$ in ischemic tissue with low $PO_2$, and better facilitate $O_2$ unloading from low-affinity Hb in RBCs |

## 25.3 Stroke

### *25.3.1 Models of Ischemic Stroke*

Several models of ischemic stroke have been used in which the MCA is occluded in rats and mice. One approach is to directly occlude the MCA either proximal or distal to the lenticulostriate arterial branches via a craniectomy to produce ischemia in striatum and cerebral cortex or only cerebral cortex, respectively. Another commonly used technique of MCA occlusion (MCAO) involves surgery in the neck to insert a filament with a slightly enlarged tip through the internal carotid artery until the tip wedges at the origin of the MCA. The filament can be withdrawn later to permit reperfusion. The filament technique prevents the need for craniectomy and produces ischemia in striatum and cerebral cortex. During occlusion of the MCA by either of these approaches, some perfusion persists through collateral arteries on the pial surface between the MCA territory and the territory supplied by the anterior and posterior cerebral arteries and thereby produces a spatial gradient of blood flow. This collateral blood flow permits the delivery of transfused HBOCs into the ischemic region.

Depending on the animal species and anesthetic, the brain normally extracts about 35–45 % of the supplied $O_2$. With moderate reductions in cerebral blood flow (CBF), increases in $O_2$ extraction help maintain $O_2$ consumption. However, global $O_2$ consumption will fall when CBF is below approximately 50 % of normal. If the reduction persists for periods of minutes to hours, sufficient numbers of neurons will die, generating an inflammatory response and eventual infarction. Boosting $O_2$ supply above 50 % of the normal value should limit the spread of infarction. Thus, enhancing $O_2$ supply with HBOCs could theoretically provide sufficient $O_2$ in the ischemic border region to prevent the spread of infarction. However, in the core of the ischemic region at a distance from anastomotic

connections, the resistance to perfusion via collateral arteries is greater than the resistance to perfusion of the border region. Hence, the ability to raise $O_2$ supply above 50 % of the normal value is more difficult as one moves away from the border region. Thus, boosting $O_2$ supply can rescue tissue in the ischemic border region near collateral arteries, but salvage of tissue in the ischemic core is more difficult.

### *25.3.2 Use of Cross-Linked Hemoglobin in Stroke*

The first cell-free Hb solutions tested in experimental models of stroke were the first generation of HBOCs in which cross-linking agents were used to stabilize the Hb tetramer to prevent dissociation into dimers that would be readily cleared by the kidney. A diaspirin cross-linking reagent was used to cross-link the two α subunits in a way that maintained Hb-$O_2$ affinity near the normal value of RBC-based Hb. This αα-cross-linked tetrameric Hb product was evaluated in the rat with both direct occlusion of the proximal MCA and with the filament model of MCAO.

Early studies with this HBOC used a hypervolemic exchange transfusion to increase plasma Hb and decrease hematocrit. The concept of hypervolemic exchange transfusion was based on earlier literature that suggested that the combination of hemodilution to decrease blood viscosity and moderate hypertension to increase perfusion pressure through collateral arteries would increase CBF in the ischemic territory. Indeed, early clinical trials of hemodilution without HBOCs during stroke were attempted, but these failed because the reduction in hematocrit was too modest to have a meaningful effect on viscosity. In addition, blood volume status was not always strictly maintained, and the induction of hemodilution was delayed. Moreover, a decrease in $O_2$ carrying capacity offsets any improvement in CBF. However, decreasing hematocrit by exchange transfusion with a HBOC should be superior because a decrease in blood viscosity could be achieved without a large decrease in $O_2$ carrying capacity.

This concept was supported by a series of studies by Daniel Cole and colleagues. They found that exchange transfusion with αα-cross-linked Hb before the onset of MCAO increased CBF during MCAO in the ischemic brain (Cole et al. 1992, 1994) and reduced the formation of cerebral edema and the subsequent infarct volume (Cole et al. 1993a, b). Increasing the plasma Hb concentration by exchange transfusion of hyperoncotic solutions that contained high Hb concentrations further reduced infarct volume and was superior to transfusion of oncotically matched solutions of albumin (Cole et al. 1996). While these studies provided a proof of concept, this work had limitations in that the transfusion occurred just before or immediately after the onset of stroke and the recovery period was often short. A subsequent study demonstrated that inducing hypervolemic hemodilation with the αα-cross-linked Hb after the onset of MCAO reduced

infarct volume and improved neurological function 3 days after transient MCAO (Cole et al. 1997).

Another group tested the effect of using exchange transfusion with αα-cross-linked Hb to decrease hematocrit to 30 % in a different focal ischemia model that combined occlusion of the common carotid artery with MCAO for different durations (Aronowski et al. 1996). They found that transfusion of this HBOC before the induction of ischemia increased the duration of focal ischemia required to produce a half-maximal infarct size from 99 to 170 min. Likewise, in a model of reversible spinal cord ischemia, transfusion of αα-cross-linked Hb increased the duration of ischemia that could be tolerated before paraplegia was observed (Bowes et al. 1994). On the other hand, transfusion was ineffective in a multifocal ischemia model in which microsphere emboli were infused into the carotid artery (Bowes et al. 1994). The lack of efficacy in this model may be related to the lack of collateral blood flow to permit delivery of the HBOC from the non-ischemic into the ischemic micro regions and to the permanent nature of the plastic microsphere occlusions.

Based on the positive results in the transient MCAO models, a clinical trial of ischemic stroke was initiated with the αα-cross-linked Hb (Saxena et al. 1999). However this trial was stopped because of safety concerns. In retrospect, the trial was not optimally designed. The volume that was transfused per body weight was considerably less than that which provided maximum protection in animals, and the plasma Hb concentration was relatively low. Moreover, the time to administration from the onset of stroke was as long as 18 h, whereas the benefit for improving oxygenation is probably limited to the first 3–6 h. Because the protocol used in the clinical trial differed substantially from that used in experimental models, it is difficult to make conclusions regarding the clinical efficacy of this product for ischemic stroke.

However, other concerns persist with the use of αα-cross-linked tetrameric Hb. Although cross-linked tetramers are not filtered by renal glomeruli, they are permeable in peritubular capillaries (Matheson et al. 2000) and can increase intestinal endothelial permeability (Baldwin 1999). Extravasation in these beds scavenges NO, produces vasoconstriction (Sampei et al. 2005; Ulatowski et al. 1996), and could have secondary effects on the brain during stroke. Transfusion of this product in rats has been reported to increase plasma endothelin levels (Gulati et al. 1995), and plasma endothelin levels were elevated in stroke patients who received the αα-cross-linked Hb (Saxena et al. 1998). Finally, a study in cats with exchange transfusion of a slightly different cross-linked tetrameric Hb failed to find an immediate benefit on intra-ischemic CBF during MCAO or early injury volume (Rebel et al. 2002). Consequently, more recent work has shifted to the use of Hb molecules with a larger molecular radius that limits extravasation and scavenging of NO in renal and splanchnic circulations.

### 25.3.3 Use of Hemoglobin Polymers in Stroke

With the use of *Escherichia coli* expression systems, polymers of recombinant Hb can be generated in quantities sufficient for transfusion. One such polymer possesses a human $\alpha$-subunit and a bovine $\beta$-subunit. Because bovine Hb utilizes chloride rather than 2,3-diphosphoglycerate as an allosteric modifier, the $P_{50}$ of this product was 17 mm Hg and was close to that of native acellular Hb. To promote polymerization, serine-9 on the $\beta$-subunit was substituted with a cysteine so that adjacent $\beta$-subunits could be linked with stable disulfide bonds. Other surface cysteines were substituted to prevent spurious disulfide bond formation. The site and angle of polymerization was such that protein folding limited polymerization to approximately seven tetramers (Bobofchak et al. 2003). Exchange transfusion of a 6 % solution of this recombinant Hb polymer, designated Polytaur, in a volume sufficient to decrease hematocrit to approximately 36 % after the onset of MCAO in mice resulted in a 66 % reduction in infarct volume (Nemoto et al. 2006). Remarkably, another recombinant Hb polymer with a $P_{50}$ of only 3 mm Hg was also effective in reducing infarct volume. In contrast, a cross-linked tetrameric Hb with a $P_{50}$ of 34 mm Hg failed to significantly reduce infarct volume in the murine model (Mito et al. 2009). Together, these studies suggest that acellular Hb that has a $P_{50}$ intermediate between that of RBC-based Hb and that of hypoxic tissue may be more effective than a high-$P_{50}$ HBOC in alleviating ischemic injury.

The benefit of low-$P_{50}$ Hb polymers during MCAO is not restricted to recombinant Hb. One such polymer, designated zero-link bovine Hb polymer (ZL-HbBv), uses a cross-linking reagent that is not incorporated into the molecule when the inter-tetramer covalent bonds are generated during polymerization. This polymer is not filtered in renal peritubular capillaries (Matheson et al. 2002), does not produce arterial hypertension or renal or splanchnic vasoconstriction (Mito et al. 2009), and does not interfere with cerebrovascular responses to endothelial- and non-endothelial-dependent vasodilators (Qin et al. 2008; Rebel et al. 2006). Exchange transfusion of a 6 % solution of ZL-HbBv polymers in the 500–14,000 kDa molecular weight range in mice after MCAO reduced infarct volume (Mito et al. 2009). Because the polymers have a $P_{50}$ of about 4 mm Hg, this finding also supports the concept that a $P_{50}$ below that of RBC-based Hb is beneficial during ischemia.

### 25.3.4 Use of Other Hemoglobin Modifications in Stroke

Storage of HBOCs in the carboxy state prevents auto-oxidation to methemoglobin and thereby extends the storage time of the product. One concern with this approach is that the carbon monoxide (CO) bound to the heme of the HBOC will limit $O_2$ carrying capacity. However, CO on the HBOC will rapidly exchange with

Hb in the RBCs and presumably other heme-containing molecules in the body (Vandegriff et al. 2008). Thus, the amount of COHb in whole blood after transfusion of an HBOC that contains COHb will depend on the moles of transfused COHb relative to the moles of Hb in the entire blood volume. After transfusion of 10 mL/kg COHb, the whole-blood COHb remained less than 3 %, indicating that $O_2$ carrying capacity is only slightly reduced (Klaus et al. 2010). This particular HBOC had polyethylene glycol (PEG) moieties attached to the Hb surface to increase the molecular radius and reduce extravasation. Interestingly, transfusion of only 10 mL/kg PEG-COHb or ZL-COHbBv after MCAO in rats reduced infarct volume despite achieving plasma Hb concentrations of only 0.6 g/dL (Zhang et al. 2012). This level of plasma Hb is considerably less than the 2 g/dL concentration that is normally associated with reduced infarct volume after exchange transfusion of non-CO HBOCs. Thus, the release of CO may actually confer protection during ischemia and increase the efficacy of the HBOC at low transfusion volumes. One way by which PEG-COHb can confer protection is by augmenting vasodilation of collateral vessels. Although PEG-COHb exerts little effect on pial artery diameter in the absence of ischemia, it has been reported to better maintain dilation during MCAO than PEG-Hb without CO and to increase perfusion in the ischemic border region (Zhang et al. 2012). Under certain conditions, released CO may also exert anti-inflammatory and anti-apoptotic effects.

Another approach is to confer the Hb molecule with antioxidant properties. Addition of nitroxide moieties to proteins confers superoxide dismutase–mimetic activity, and infusion of polynitroxylated albumin during or 2 h after reperfusion from transient MCAO in rats reduces infarct volume (Sugawara et al. 2001). This technology has been adapted to HBOCs and has been found to inhibit endothelial leukocyte adhesion in a model of endothelial dysfunction (Saetzler et al. 1999). Furthermore, a polynitroxylated PEGylated Hb (PNPH) has been found to reduce brain injury in a model of polytrauma in which traumatic brain injury is followed by hemorrhagic hypotension (Shellington et al. 2011). This product also can reduce infarct volume caused by transient MCAO when transfused at relatively low volumes (10 mL/kg).

In addition to heme binding of NO, the Hb molecule can carry NO in an S-nitrosylated form. S-nitrosylation occurs on the $\beta$93 cysteine on Hb, and this process depends on the oxygenation state of Hb (McMahon et al. 2002). Deoxygenation of Hb as it passes through the microcirculation causes the release of NO from the cysteine. Greater release of NO under hypoxic conditions is expected to promote vasodilation. An S-nitrosylated HBOC has been produced with human PEG-Hb. In a thrombotic model of permanent MCAO, transfusion of a relatively low dose of 0.4 mL/kg of a 6 % S-nitrosylated PEG-Hb solution (24 mg/kg) resulted in a moderate decrease in infarct volume, whereas an equivalent dose of non-nitrosylated PEG-Hb had no effect (Kawaguchi et al. 2009). However, the effective dose range was narrow, as doses of 0.08 and 2 mL/kg produced no protective effect.

S-nitrosylated PEG-Hb has also been evaluated in a model of incomplete global cerebral ischemia with 10 min of bilateral carotid artery occlusion (Otani

et al. 2004). This model produces alterations in synaptic strength in hippocampus without necessarily inducing cell death. Interestingly, delayed transfusion of 2.5 mL/kg (250 mg/kg) nitrosylated PEG-Hb at 24 or 48 h of reperfusion improved recovery of long-term potentiation in the hippocampus at 96 h of reperfusion. The delayed transfusion was also associated with an attenuation of inducible NO synthase expression and an augmentation of endothelial and neuronal NO synthase expression. Thus, release of NO from small amounts of an S-nitrosylated HBOC can exert direct protective effects that enhance functional recovery of synapses and that might suppress post-ischemic inflammation. Whether an S-nitrosylated PEG-Hb with a long circulating half-life has greater potency than a traditional NO donor molecule remains to be determined.

Another approach is to encapsulate Hb in liposomes that are decorated with PEG on their surface to reduce aggregation and uptake by the reticuloendothelial system. The liposomes permit inclusion of the allosteric modifier inositol hexaphosphate to increase the $P_{50}$ to 40–45 mm Hg. In a model of thrombic-induced MCAO in rats, transfusion of 2 or 10 mL/kg of the liposome suspension containing the equivalent of 6 g/dL Hb reduced infarct volume (Kawaguchi et al. 2007). Thus, this HBOC is effective in a model of permanent focal ischemia. Similar findings were observed in a primate model of transient MCAO in which cortical $O_2$ consumption was improved and acute injury was ameliorated (Kawaguchi et al. 2010). Interestingly, lowering the $P_{50}$ to 10 mm Hg by decreasing the inositol hexaphosphate provided greater protection to rats after permanent MCAO than did liposomal Hb with high $P_{50}$ and reduced the transfusion volume required to less than 2 ml/kg (Fukumoto et al. 2009). Similar findings were reported with transient cochlear ischemia in the gerbil (Okada et al. 2012). In contrast to the $P_{50}$ of the HBOC, allosteric modifiers that increase the $P_{50}$ of RBC-based Hb are protective in MCAO (Watson et al. 1997). Thus, facilitation of $O_2$ diffusion between RBCs and hypoxic tissue may be optimal when RBCs have a high $P_{50}$ and liposomal Hb or free Hb in the plasma has a $P_{50}$ intermediate between the RBC Hb $P_{50}$ and the tissue $PO_2$.

### *25.3.5 Hemoglobin Toxicity to Neurons*

Native Hb is toxic to neurons, and this toxicity is exacerbated by loss of constitutive heme oxygenase-2 in neurons. Thus, Hb toxicity is thought to be related to excessive release of free heme, which is known to augment formation of reactive oxygen species. Because the blood–brain barrier becomes leaky after stroke, one concern is that extravasation of an HBOC during stroke may augment neuronal cell death. However, the glutaraldehyde-polymerized bovine Hb product HBOC-201 has been reported not to be toxic to cultured neurons after 24 h exposure at concentrations typically attained in plasma (Ortegon et al. 2002), possibly because this polymeric Hb degrades more slowly than native Hb and therefore releases free heme more slowly. Remarkably, the PNPH product actually protects cultured

neurons from native Hb and from glutamate exposure, presumably because of its superoxide dismutase–mimetic activity (Shellington et al. 2011). Thus, this product may be safe to administer whether the stroke is ischemic or hemorrhagic in origin. Because imaging of the stroke patient to exclude hemorrhagic stroke can delay administration of tissue plasminogen activator for clot dissolution, early administration of PNPH before imaging could theoretically serve as a safe therapeutic to preserve neuronal viability until reperfusion is established by clot removal.

In summary, studies in a variety of animal models of stroke suggest that HBOCs may be a useful therapy to limit damage. However, most studies used early transfusion, and it is unclear how effective HBOCs might be when administered after more clinically relevant delays from the onset of stroke symptoms. Nevertheless, some of the special preparations may work during the reperfusion period by mechanisms in addition to those that improve oxygenation and could thereby have a longer window of opportunity.

## 25.4 Myocardial Ischemia

### *25.4.1 Ability of HBOCs to Meet Myocardial Metabolic Needs*

Because of the heart's very high $O_2$ consumption, restoring cardiac function after coronary artery occlusion requires delivery of a large amount of $O_2$. One question is whether HBOCs alone are capable of sustaining myocardial $O_2$ consumption at a level sufficient for normal cardiac function. Early work suggests that this may be the case. Near-complete exchange transfusion with polymerized bovine Hb to decrease hematocrit to less than 3 % was able to keep unanesthetized rats alive over a 4 h observation period; during this time blood pressure remained elevated and brain glucose consumption was maintained (Waschke et al. 1993). To determine if perfusion with HBOC-201 can sustain cardiac function in the absence of RBCs in the heart of a large animal, researchers occluded a coronary artery in anesthetized pigs and perfused oxygenated HBOC-201 distal to the occlusion (te Lintel et al. 2010). Without perfusion, regional cardiac function deteriorated within 3 min, whereas distal perfusion with HBOC-201 for 3 min with adequate flow could fully sustain cardiac function and prevent increases in venous adenosine concentrations. Thus, this Hb polymer with a $P_{50}$ of 40 mm Hg is capable of delivering $O_2$ sufficient to sustain aerobic metabolism and cardiac function in the absence of RBCs, at least for brief periods of time.

### 25.4.2 Coronary Artery Occlusion

Other studies have examined the effects of HBOC transfusion during coronary artery occlusion without removing most of the RBCs. In one study, anesthetized dogs received an exchange transfusion of approximately 6 mL/kg of saline or HBOC-201 before 90 min of occlusion of the left anterior descending coronary artery (Caswell et al. 2005). Whereas the reduction in intraischemic myocardial perfusion was similar between groups, the HBOC-201–treated group had improved recovery of myocardial contractile function, reduced leukocyte infiltration, and decreased infarct size at 4.5 h of reperfusion. One interpretation is that the HBOC buffers the fall in subendocardial $PO_2$ and that reperfusion injury is attenuated as a result of the mitigation of ischemic severity by the HBOC. Alternatively, the HBOC may directly improve endothelial function and directly mitigate reperfusion injury.

In another study on anesthetized dogs, HBOC-201 was transfused after the onset of myocardial ischemia (George et al. 2006). Here, occlusion was incomplete so that the HBOC could be delivered into the ischemic territory. Transfusion of 1 g/kg HBOC-201 after the onset of occlusion attenuated the increase in left ventricular end-diastolic pressure and the decrease in cardiac function. HBOC-201 decreased the intraischemic coronary vascular resistance. This decrease possibly occurred secondary to HBOC-201 preventing the increase in left ventricular diastolic pressure from causing an increase in the zero flow-pressure intercept that occurs with increased end-diastolic pressure. When the heart was concurrently paced at an increased rate for 195 min during the induced coronary stenosis, infarct volume measured at 3 h of reperfusion was markedly attenuated in the group transfused with HBOC-201 during ischemia. Together, these studies suggest that HBOC-201 can be beneficial in cases of acute, incomplete coronary arterial occlusion; in cases of acute, complete occlusion followed by reperfusion, HBOC-201 might also limit the inflammatory response associated with reperfusion injury.

One potential concern with the use of HBOCs during ischemia is that they could scavenge endothelial-derived NO, produce vasoconstriction, and thereby exacerbate the ischemia. However, these two studies did not find an adverse effect of HBOC-201 on myocardial perfusion. It should be noted that NO plays a greater role in vasodilation in large coronary arteries while other endothelial-dependent vasodilators are thought to predominate in the smaller resistance arterioles. With stenosis of large coronary arteries, sustained dilation of downstream resistance arterioles may be less dependent on NO and thus affected less by potential HBOC scavenging of NO.

Although HBOCs may have few adverse effects on downstream vascular resistance, use of an HBOC that can release NO might exert some additional benefit. (Asanuma et al. 2007) have studied the effect of S-nitrosylated PEG-Hb on coronary blood flow in the canine heart. Rather than producing a coronary occlusion, they perfused the left anterior descending coronary artery at a low perfusion pressure sufficient to reduce blood flow to 33 % of normal. Adding

either S-nitrosylated PEG-Hb or nitroglycerin to the perfusion increased coronary blood flow, but the combination had no additional effect. Adding PEG-Hb alone had no benefit. Thus, the NO-donating ability of S-nitrosylated PEG-Hb appears to be able to produce additional coronary dilation under conditions of reduced perfusion pressure. Whether such a benefit occurs with severe stenosis remains to be determined.

Another alternative approach is to use a carboxy HBOC. In a model of coronary occlusion in the rat, transfusion of human PEG-COHb before occlusion was superior to transfusion of oxy-PEG-Hb in reducing infarct size (Vandegriff et al. 2008). Whether a carboxy HBOC will be more effective after the onset of occlusion is unclear.

In clinical practice, the delivery and efficacy of an HBOC under conditions of myocardial ischemia will depend on the degree of coronary stenosis and the amount of collateral flow. These factors are likely to be highly heterogeneous in a clinical population with assorted risk factors such as hyperlipidemia, hypertension, and diabetes. Thus, those with risk factors for myocardial ischemia may also be those with poor collateral blood flow and those who will receive less benefit from HBOCs. Moreover, because the use of the early generations of HBOCs in the setting of hemorrhagic shock was associated with myocardial adverse effects (Natanson et al. 2008), concerns persist that the use of HBOCs could exacerbate myocardial injury. The newer formulations of S-nitrosylated and carboxy HBOCs will need to be evaluated in animal models of pre-existing risk factors.

### *25.4.3 Preservation During Complete Myocardial Ischemia*

In a different myocardial application, HBOCs have been evaluated as an additive to cardioplegic solutions. Polymerized Hb with a $P_{50}$ of 5–9 mm Hg was obtained from human placenta and added to a cardioplegic solution that was perfused through rat heart. The heart was then stored at 4 °C for 9–14 h. After normothermic reperfusion and washout of the HBOC, the myocardial inflammatory response was suppressed and cardiac function was improved (Wei et al. 2011a). However, the effective concentration range in the cardioplegic solution was very narrow; 0.3 g/dL was effective whereas 0.1 and 0.5 g/dL were ineffective (Wei et al. 2011b). In contrast, another study showed that a lower concentration of polymerized placenta Hb (0.1 g/dL) was effective in preserving the heart and limiting oxidative damage to mitochondria (Li et al. 2010). An optimal concentration and therapeutic range in human heart could be quite different from those in isolated rat heart.

## 25.5 Ischemia in Other Tissue

A limited number of studies have investigated the use of HBOCs in organs other than brain and heart. With 4 h of ischemia in hamster striated skin muscle, transfusion of crosslinked tetrameric Hb before reperfusion reduced leukocyte adherence during reperfusion and reduced injury (Pickelmann et al. 1998). Transfusion of liposome-encapsulated Hb after the onset of 70 min of hindlimb ischemia in rats modestly attenuated the decrease in intracellular pH measured by phosphorus magnetic resonance spectroscopy during ischemia and appeared to better preserve muscle mass at one week of recovery (Kurita et al. 2012).

In a model of liver ischemia in the rat, exchange transfusion with dextran or HBOC-201 was superior to exchange transfusion with Ringer's lactate solution in establishing reperfusion, and HBOC transfusion was superior to dextran transfusion in improving tissue $PO_2$ during reperfusion (Zapletal et al. 2009). However, the improvement in circulating liver enzymes with HBOC-201 transfusion was not superior to that with dextran transfusion. Longer recovery times and use of histopathology may be needed to discern a distinct advantage of the HBOC.

HBOCs have also been considered for use in reducing ischemic-reperfusion injury in skin grafts. However, one study failed to find a reduction in necrosis in rat island groin skin flaps after transfusion with polymerized Hb (Hofeling et al. 2006). However, this HBOC product (Oxyglobin) has a significant amount of non-polymerized Hb that may produce adverse effects.

Overall, more work is needed to better define the clinical conditions of peripheral ischemia under which transfusion of HBOCs may be beneficial and to refine clinically meaningful endpoints in these types of experimental studies.

## 25.6 Conclusions

A considerable literature indicates that HBOCs hold promise as an oxygen therapeutic under ischemic conditions, and some reports suggest that they might also be beneficial during reperfusion. The HBOC can be beneficial during ischemia if there is sufficient collateral blood flow into the ischemic region and if transfusion produces critical concentrations in the plasma. The exact mechanisms by which ischemic tissue is rescued have not been elucidated but may be related to augmented $O_2$ transport to the microcirculatory exchange site, improved homogeneity of $O_2$ flux among capillaries, and facilitation of $O_2$ flux from RBCs and to the endothelium. Moreover, some newer HBOC preparations, such as those that contain COHb, polynitroxylated Hb, S-nitrosohemoglobin, and encapsulated Hb, appear to enhance efficacy and may permit the use of smaller transfusion volumes, possibly by stabilizing endothelial function and limiting inflammation.

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# Part VI
# Preclinical Evaluations of HBOCs

# Chapter 26
# Pre-clinical Evaluation of Hemoglobin Based Oxygen Carriers: Animal Models and Biomarkers

**Paul W. Buehler and Felice D'Agnillo**

## 26.1 Introduction

Preclinical testing is designed to demonstrate general proof of concept, but primarily reasonable safety risks to human subjects in various phases of clinical evaluation and post regulatory approval. Preclinical testing of hemoglobin-based oxygen carriers (HBOCs) has faced important challenges particularly related to extrapolating safety assessments in normal healthy animals to a heterogeneous population of subjects with disease state complications and underlying co-morbidities (Silverman and Weiskopf 2009a). The adverse event profile of some HBOCs in human clinical trials combined with the lack of corroborating overt toxicological response in normal animals highlights a clear disconnect between preclinical safety results and clinical safety outcomes (Buehler and Alayash 2004). This has suggested the need for more refined, novel and innovative approaches for assessing HBOC safety in humans (Natanson et al. 2008).

The present chapter discusses preclinical approaches that focus on animal species with potential relevance to humans in terms of pharmacokinetics, pharmacodynamics and physiologic response to HBOCs, animal models of disease, as well as biomarkers of Hb exposure, inflammation, oxidative stress and end organ

The findings and conclusions in this chapter have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any agency determination or policy

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toxicity. Proposed mechanisms underlying the toxicity of Hb/HBOCs with particular emphasis on heme- and/or iron-mediated oxidative stress will also be discussed.

## 26.2 Animal Models of Efficacy (Proof of Concept)

Models of efficacy in animals can be used to establish a proof of concept to demonstrate physiologic/pharmacologic effect and to suggest feasibility in support for use in human specific indications. Proof of concept together with safety pharmacology and toxicology studies provides a basis for progression into clinical trials. However, the main purpose of pre-clinical animal testing remains the assurance of safety in human subjects in all phases of clinical trials. Numerous proof of concept models have been studied in the non-clinical evaluation of HBOCs to establish physiologic effects on tissue blood flow and oxygenation as well as efficacy in primary disease states for which HBOCs could be indicated.

### *26.2.1 Traumatic Hemorrhage*

Animal models of either targeted blood volume removal and/or pressure controlled hemorrhage have been used in combination with tissue injury (tissue damage, bone fracture, brain injury and chest injury) to study the early and late physiologic effects of trauma in combination with blood loss. Several animal species including rodents (mice and rats) (Makley et al. 2010; Schmand et al. 1994; Capone et al. 1995), guinea pigs (Swafford et al. 2003), dogs (Woodman et al. 1986; Capone et al. 1996), swine (Manning et al. 2000; Hess et al. 1993; Stern et al. 2000, 2009), sheep (Frithiof et al. 2006) and non-human primates (Pretorius et al. 1987) have been evaluated. Each species provides advantages and disadvantages in the study of hemorrhage and trauma. For example, advances in transgenic manipulation and increasing commercial availability of specialized mice provide the advantage of studying the role of endothelial dysfunction (Yu et al. 2010), and coagulopathy (Pergolizzi et al. 2006). With advancements in microsurgery, long term implantable pumps, telemetry probes and specialized catheters previous technical limitations with mouse studies are more realistic. Swine are the primary non rodent species, reported in the literature to evaluate HBOC resuscitation from traumatic hemorrhage (Buehler and Alayash 2004). Advantages of this species may include similar cardiovascular and systemic hemodynamic responses to human and established models of arterial injury induced uncontrolled hemorrhage (Stern et al. 1993, 2000). Disadvantages suggested by unpublished observational data gathered from multiple studies indicate that the species can tolerate long durations with low hematocrits ($\sim$3 g/dL of hemoglobin) and may therefore not extrapolate well to human transfusion or be predictive of survival. Additionally, pulmonary

macrophage accumulation in swine tends to exaggerate pulmonary hypertensive response, not observed in many other species. From a physiology and pharmacology perspective, these models can provide valuable insight into the impact of HBOCs on short and long term physiological consequences of traumatic hemorrhage.

### *26.2.2 Local Ischemia*

Several HBOCs have been proposed for use in ischemic stroke, myocardial infarction and coronary artery by-pass graft (CABG) surgery (Buehler and Alayash 2004). Diaspirin cross-linked hemoglobin (DCLHb) demonstrated efficacy in an ischemic stroke model of middle cerebral artery occlusion in rats (Cole et al. 1992, 1993). In stroke patients, however, DCLHb treatment worsened ischemic stroke (Saxena et al. 1998, 1999). This disconnect is likely related to the inability to sufficiently extrapolate ischemic risk assessment in healthy animals subjected to periods of ischemia to humans with multiple factors contributing to vascular disease. Therapeutics intended for use in stroke are tested according to extensive preclinical efficacy guidelines proposed by the first Stroke Therapy Academic Industry Roundtable (STAIR) in 1999 and updated in 2009 (1999, Fisher et al. 2009). While these guidelines have not specifically been applied to HBOCs, a clear understanding of the potential for HBOCs to enhance local toxicity in stroke and other ischemic conditions may be warranted.

### *26.2.3 Sickle Cell Disease*

Conceptually, HBOC mediated delivery of $O_2$ as well as delivery of low level carbon monoxide during vaso-occlusion may be promising in a disease state with limited therapeutic options. From a pharmacologic perspective, HBOC treatment may target reversal or prevention of vaso-occlusive crisis. Options for pre-clinical study exist in transgenic mice, although these models are not widely available. Two examples of models used to evaluate toxicity and proof of concept include the S+S Antilles and BERK transgenic sickle cell mouse strains. The S+S Antilles strain are based on C57BL/6 mice but express human $\alpha$, $\beta^{s}$ and $\beta^{s\text{-Antilles}}$ globin transgenes with approximately 42 % of $\beta$ globin chains expressed as $\beta^{s}$ and approximately 36 % as $\beta^{s\text{-Antilles}}$ (Belcher et al. 2005, 2006; Fabry et al. 1995). BERK mice express human $\alpha$, $\beta^{s}$ globin transgenes and represent the most severe model of disease (Belcher et al. 2006; Paszty et al. 1997). Both transgenic strains experience RBC congestion, inflammation, ischemia and tissue infarction. However, the S+S Antilles mouse is a less severe model and provides an opportunity to understand oxidative and inflammatory mechanisms underlying the pathology of sickle cell disease. Published evaluations suggest that appropriate study designs should rely on

two independent transgenic mouse strains as differing models demonstrate strengths and weaknesses (Belcher et al. 2006). However, there are numerous safety based questions associated with extracellular Hb exposure in sickle cell disease in part caused by extracellular Hb exposure to the systemic circulation and highly vascularized tissue. Additionally, sickle cell disease patients experience renal insufficiency and renal failure, which may be exacerbated by HBOCs.

### *26.2.4 Pre-clinical Evaluation*

The regulations of biologic products within the United States are governed by the Public Health Service act as well as the Food Drug and Cosmetic acts. The Code of Federal Regulations (CFR) for biologics (21 CFR 601.2 (a)) states that manufacturers, in order to obtain a license for marketing, must submit pre-clinical data to support safety, purity and potency. Under 21 CFR 312.23 (a) (8), the kind, duration and scope of animal and other tests required varies with the duration and nature of the proposed clinical investigation. Guidance on the type and scope of nonclinical safety studies are recommended by the International Conference on Harmonization (ICH) based on the recommendations of representatives from industry, government and academia. Additionally, guidance documents are drafted within the U.S. Food and Drug administration (FDA) to reflect the current FDA thinking on a particular topic.

## 26.3 Safety Pharmacology and Toxicology

Safety pharmacology studies typically evaluate functional indices of toxicity in major organ systems. These include physiologic/clinical monitoring of the cardiovascular, renal, pulmonary, gastrointestinal and central nervous systems to assess potential safety concerns in specific organ systems. For the pre-clinical evaluation of HBOCs, safety pharmacology studies can be useful to establish HBOC specific systemic and pulmonary blood pressure effects (vascular), monitoring for hemoglobinuria (renal), confusion and ataxia (CNS), vomiting/diarrhea (gastrointestinal) and electrocardiography (cardiac). The findings from safety pharmacology studies may help focus on organ systems and toxicity endpoints.

Toxicology studies performed to support human safety follow good laboratory practice (GLP) regulations outlined in 21 CFR part 58. This regulation identifies the conduct of non-clinical studies intended to support safe human use of novel therapeutics (drugs and biologics). Typical toxicology studies are performed in normal animals of both genders to understand the relationship between dosing levels, pharmacokinetic exposure and systemic as well as local toxicity. Studies are designed to capture the intended clinical administration route, dosing regimen and recommended duration of exposure to support the desired clinical trial

duration and licensure. The primary guidance documentation for the scope and duration incorporated in study designs are outlined in the ICH S6(R1) for biotechnology derived pharmaceuticals and the ICH M3(R2) on non-clinical safety studies for the conduct of human clinical trials of pharmaceuticals. Both guidance documents may apply to the non-clinical development of HBOCs. Under some circumstances animal models of disease may help to understand the impact of a given product candidate on toxicity. To maximize the relevance and prevent repetition in non-clinical studies the test material used should be comparable to the final product used in clinical studies and that proposed for licensure.

HBOCs have presented a challenge to the paradigm of standard safety testing. Parameters evaluated in animal studies have in several circumstances not adequately predicted adverse outcomes in later phases of clinical testing (Silverman and Weiskopf 2009a, b; Buehler and Alayash 2004). There are several factors that may account for this including; *Timing of toxicological assessment*—Early time points (within 24 after dosing) of toxicological evaluation are not routinely performed and therefore, indicators of acute responses in tissue may be undetected. *Sensitivity of standard toxicology*—standard testing typically does not employ specialized tissue staining techniques to identify indicators of oxidative stress, heme or iron overload, and inflammation in vascular tissue of major blood vessels or within the vasculature of critical organ systems (heart, brain and kidneys). *Specialized models*—animal models relevant to exacerbation of HBOC toxicity such as antioxidant depletion, early endothelial dysfunction, advanced endothelial dysfunction (atherosclerosis) and renal impairment have not been routinely used to evaluate safety.

## 26.4 Experimental Approaches to Assess Pre-clinical Safety of HBOCs

### *26.4.1 Species Selection Based on Natural Antioxidant Status*

Selection of animal species for assessing pre-clinical safety is often determined by the pharmacokinetic, pharmacologic or physiologic profile of the species and relevance to humans. Pro-oxidative response in vivo may be a relevant pharmacologic and physiologic predictor of human toxic response to HBOCs. The initiation of oxidative toxicity may be acute or long term, but is likely dependent on circulating as well as tissue antioxidant status. Non-human primates and guinea pigs are similar to humans in terms of their overall plasma and tissue antioxidant capabilities (Chatterjee 1973; Nandi et al. 1997). All three species are unable to produce ascorbic acid (AA) due to an evolutionary loss of L-gulonolactone-$\gamma$- oxidase (LGO), which is the rate limiting enzyme in AA synthesis. Therefore, these species must rely on dietary intake rather than hepatic production, common

to all other mammals (Chatterjee 1973). In the case of small animals, the guinea pig additionally has up-regulated cellular antioxidant systems (superoxide dismutase and catalase) in agreement with human systems (Nandi et al. 1997). Guinea pig and human red blood cell (RBC) reductive capacities have also been suggested to be similar (Su et al. 2006). Thus, based on similarities in antioxidant capabilities to humans, the guinea pig could be a relevant small animal species to consider in the pre-clinical studies of HBOC candidates. To date, the rat has been the most commonly used rodent species in pre-clinical HBOC toxicology; however, rats have very different tissue and plasma antioxidant profiles compared to humans. Rats generate AA at a rate of 38 μg AA/mg microsomal protein/hour and much higher amounts when subjected to physical stressors (Chatterjee 1973). Other models such as transgenic LGO-knockout mice could be useful for identifying oxidative stress safety signals after administration of HBOCs. As a critical small molecule responsible for limiting ferric Hb formation, AA along with circulating RBC reductive capacity may be important to predicting HBOC oxidation and tissue exposures to ferric Hb.

### *26.4.2 Antioxidant Depletion*

Endogenous antioxidant systems play a major role in mitigating the oxidative side-effects of HBOCs. Animal models of antioxidant depletion may therefore be useful for identifying toxicities that might not otherwise be detectable in normal healthy animals with intact antioxidant defenses. A number of approaches have been employed to deplete endogenous antioxidant status in cell culture and in animals particularly for glutathione (GSH), the most abundant non-protein cellular thiol. GSH maintains cellular redox status and regulates a number of detoxification reactions. The majority of these processes generate oxidized GSH (GSSG), which is reduced back to GSH by glutathione reductase and NADPH from the pentose phosphate shunt. GSH depletion occurs in a number of clinical settings relevant to the intended application of HBOCs including ischemia–reperfusion, sepsis, organ transplantation, and myocardial conditions (Franco et al. 2007; Circu and Aw 2008). One of the most popular chemical approaches used to deplete intracellular GSH involves using buthionine-[S,R]-sulfoximine (BSO), an inhibitor of gamma-glutamylcysteine synthetase the rate-limiting step in GSH synthesis. Endothelial cells pretreated with BSO were found to be remarkably more sensitive to the cytotoxicity induced by the redox cycling of Hb and HBOCs (D'Agnillo 2004; D'Agnillo and Alayash 2001). In guinea pigs, intraperitoneal injection of BSO at a dose of 0.8 g/kg reduced GSH levels by 50–75 % in liver, heart, and kidney after 24 h consistent with effects observed in other animal species. Preclinical models of antioxidant depletion may be used to compare the oxidative toxicity of different HBOCs, and, may at the same time offer insight into potential protective antioxidant strategies.

### 26.4.3 Vascular Dysfunction

The vascular endothelium plays a key role in determining the overall response to HBOCs. The publically recorded data on adverse event profiles in clinical studies suggest that Hb-induced vascular injury is a major contributor to human toxicology (Silverman and Weiskopf 2009a, b). Studying the origin of these events has focused extensively on the potential role of nitric oxide (NO) scavenging and vasoconstriction in the processes of early and late stage toxicity while only limited studies have emphasized heme-induced oxidative stress. In vitro studies largely describe underlying mechanisms of cytotoxicity caused by interaction of heme and potentially reactive oxygen species (D'Agnillo and Alayash 2001, 2002). The balance between nitric oxide and superoxide anion in the vasculature can be disrupted in the presence of cell free Hb in favor of more powerful oxidants such as peroxynitrite and $H_2O_2$. It is likely that both decreased NO vascular function and heme interactions within the vasculature, particularly in conditions of decreased vascular compliance and at locations of plaque formation, exacerbate endothelial/vascular disease progression. In normal animals, HO-1 induction has been demonstrated within the tunica adventitia and media of large vessels (Buehler and D'Agnillo 2010). HO-1, CD163 and iron have been co-localized with areas of vascular tissue and suggest heme localized exposures do occur (Butt et al. 2011) (Fig. 26.1). These vascular regions have also been associated with the disruption of endothelial tight junctions and barrier dysfunction in the blood brain barrier of guinea pigs (Butt et al. 2011) (Fig. 26.2a). Several mechanisms have been proposed to explain the vascular toxicity of Hb and HBOCs including; (i) the liberation of highly lipophilic heme from oxidized Hb which can damage endothelial plasma membranes and potentially accumulate intracellularly (ii) accumulation of Hb or HBOCs into subendothelial spaces followed by uptake and subsequent activation of pericytes/perivascular macrophages leading to oxidative stress (iii) redox reactions driven by intact ferric Hb (e.g. ferryl species) triggering a localized oxidative insult to endothelium, and (iv) excessive Hb and heme breakdown resulting in free iron-driven oxidation reactions. Endothelial disease progression models using high fat diet supplementation, long term hypertension and diabetic animals have been established in the study of endothelial dysfunction. A mouse model of high fat dietary supplementation and NO function was recently studied with regards to Hb and HBOCs (Yu et al. 2010). This study demonstrated the adverse hemodynamic effects of a dysfunctional vascular system could be off-set by pre-dosing with inhaled NO.

### 26.4.4 Sepsis and Endotoxemia

Sepsis and other proinflammatory conditions are generally accompanied by overproduction of reactive oxygen and nitrogen species (ROS/RNS), antioxidant depletion, and inflammation. Increased lipid peroxidation and marked depletion of antioxidants such as ascorbate, vitamin E, and GSH are observed in clinical and

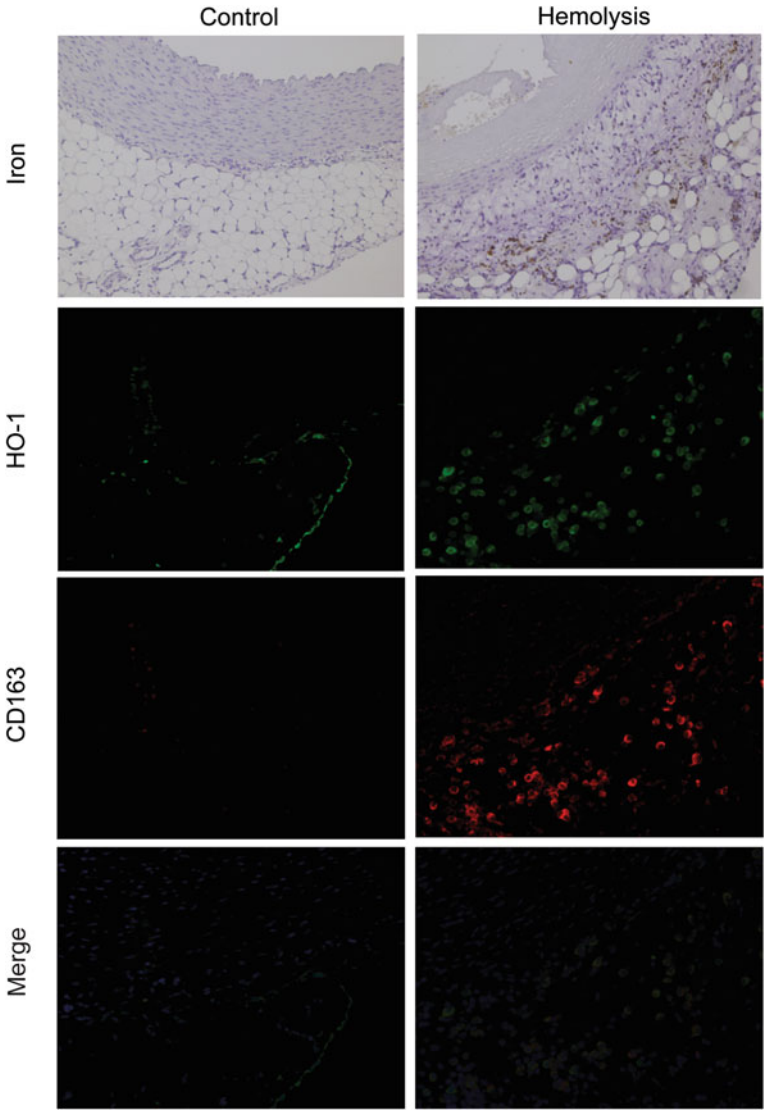
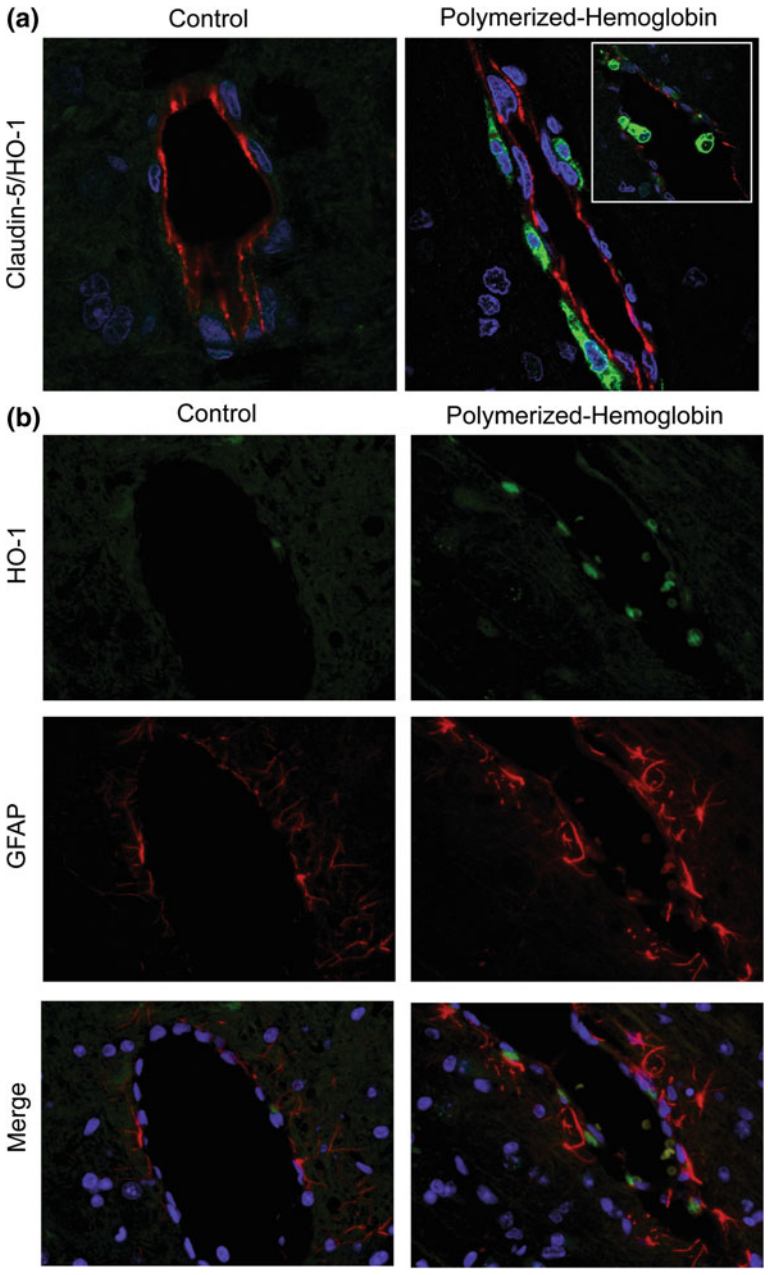



**Fig. 26.1** Peripheral vascular tissue injury—aortic adventitia blood vessels following hemolysis in guinea pigs. Areas of iron, HO-1 and CD163 co-localization are observed suggesting hemoglobin uptake within CD163 positive macrophages. Iron is shown as brown staining regions following Perls-DAB immunohistochemistry. HO-1 immunofluorescence is demonstrated following incubation with rabbit polyclonal anti-HO1, rinsed and incubated with Alexa-Fluor 488-conjugated (*green*) secondary antibody. CD163 immunofluorescence is demonstrated following incubation with mouse anti-CD163, rinsed and incubated with Alexa-Fluor 555-conjugated (*green*) secondary antibody. The merged images are shown with Hoechst positive staining nuclei (*blue*). Original magnification ×200 (Adapted from Ref. (Baek et al. 2012), supplemental data Fig. 26.4)


(a)
Control
Polymerized-Hemoglobin
Claudin-5/HO-1
(b)
Control
Polymerized-Hemoglobin
HO-1
GFAP
Merge

◀ **Fig. 26.2** Blood brain barrier dysfunction—relationship between HO-1, a marker of hemoglobin exposure, and claudin-5, a transmembrane tight junction protein, and GFAP, marker of astrocyte activation. In polymerized hemoglobin-transfused animals, HO-1-positive pericytes/perivascular macrophages lined the walls of vessels with (**a**) diffuse claudin-5 staining and (**b**) enhanced GFAP reactivity. In control animals, HO-1 negative vessels showed intact claudin-5 staining and minimal GFAP activation. Brain sections were stained using antibodies to HO-1, claudin-5, GFAP and detected using relevant Alex Fluor 488 (HO-1, *green*) and Alex Fluor 555 (CL5 and GFAP, *red*)—conjugated secondary antibodies. Nuclei were counterstained with Hoechst 33342 (*blue*). Laser scanning confocal microscopy; original magnification, ×700 (**a**). Double-label epifluorescence microscopy; original magnification, ×400 (**b**)

experimental sepsis (Bahl et al. 2011; Rinaldi et al. 2009). Sepsis is often associated with endothelial dysfunction characterized by alterations of vascular tone, increased endothelial adhesiveness and permeability, and coagulation defects (Bahl et al. 2011; Huet et al. 2011). Several studies have reported that cell-free Hb or HBOCs exacerbates the pathophysiology of sepsis or endotoxemia (Su et al. 1997; Krishnamurti et al. 1997). Cell-free Hb also enhances the inflammatory response to lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria and the primary mediator of gram-negative sepsis (Bodet et al. 2007). The synergism between Hb and LPS has been partly attributed to the ability of Hb to physically interact with LPS (Bahl et al. 2011; Kaca et al. 1994, 1995; Howe et al. 2008). Specific binding sites on Hb for LPS have recently been identified (Bahl et al. 2011). The redox cycling of various Hbs by a low level of oxidative stress, and not their direct interaction with LPS, has been shown to dramatically enhance LPS-induced apoptosis (D'Agnillo 2004). Sepsis and/or endotoxemia activate tissue resident and circulating inflammatory cells creating an oxidative microenvironment that could exacerbate the pro-oxidant reactions of Hb in the vicinity of endothelial cells (Fig. 26.3). Studying the safety of HBOCs in models of sepsis or endotoxemia could provide much needed information on a potentially key and under-researched area in the field of HBOCs.

## 26.5 Tissue Markers of Heme and Hemoglobin Exposure

### *26.5.1 Heme Oxygenase, Iron and Ferritin*

Heme oxygenase (HO) catalyzes the degradation of heme to biliverdin, an intermediate in the production of bilirubin, ferrous iron and carbon monoxide (Abraham and Kappas 2008). Of the two main isoforms, HO-2 is constitutively expressed, while HO-1 is induced by various stimuli including heme, lipopolysaccharide (LPS), hypoxia, and heavy metals (Abraham and Kappas 2008). HO-1 induction is primarily considered a protective response although, under some settings, HO over expression may have deleterious consequences (Chen and Regan 2007; Koeppen et al. 2004). Renal HO-1 induction was found to correlate with the extent of HBOC oxidation and in vivo antioxidant status (Butt et al. 2010).

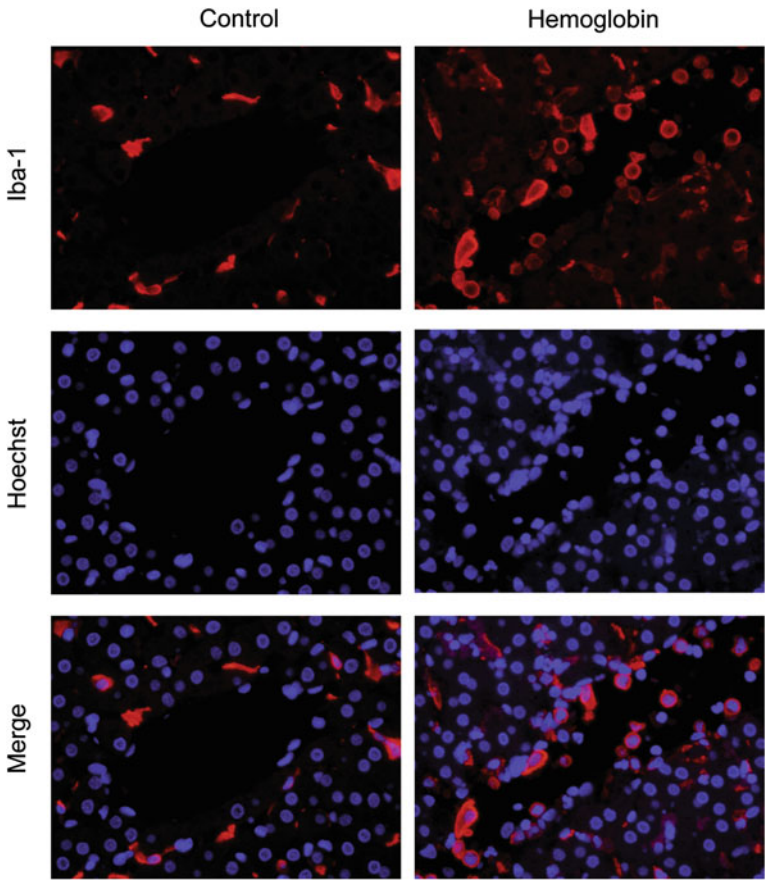



**Fig. 26.3** Endotoxemia, hemoglobin and inflammation—liver inflammation in guinea pigs infused with hemoglobin and LPS alone or in combination. Images show the combination of hemoglobin and LPS produced activation of resident macrophages and monocytic infiltration in blood vessels. Liver sections were stained using an antibody to iba-1, a marker of monocytes/macrophages, and detected using an Alex Fluor 555 -conjugated secondary antibody. Nuclei were counterstained with Hoechst 33342. Original magnification, ×400

Enhanced perivascular HO-1 expression was observed in cerebral blood vessels that showed altered tight junction integrity, increased astrocyte activation, and oxidative end products in guinea pigs transfused with polymerized Hb. The assessment of HO activity and HO-1 expression as a biomarker of Hb exposure and oxidative stress may prove useful in evaluating the responses to HBOCs. Ferritin is the main intracellular iron storage protein and composed of 24 subunits of ferritin heavy (H-ferritin) and light (L-ferritin) chains. An acute response to short-term iron stress is usually accompanied by H-ferritin induction while L-ferritin expression is typically associated with long-term iron storage (Hintze and Theil 2006). Together, ferritin and its breakdown product, hemosiderin,

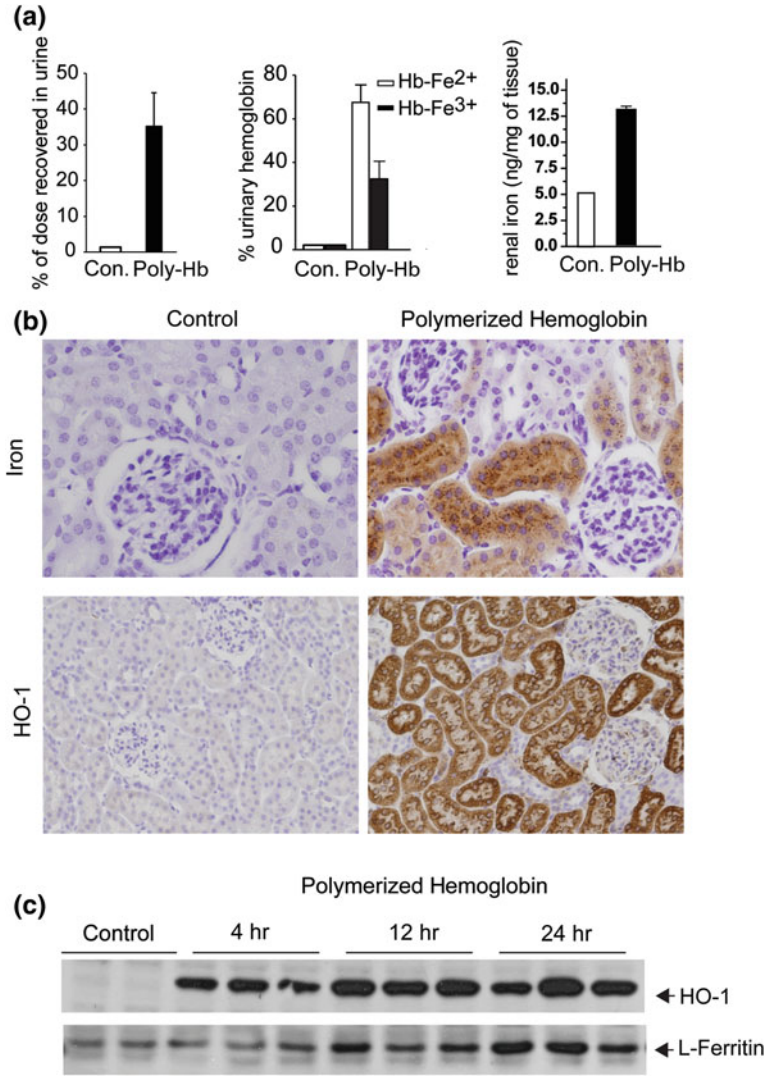



**Fig. 26.4** Renal response to polymerized hemoglobin—Guinea pigs were exchange transfused with polymerized hemoglobin. (**a**) Analysis of urine samples for percent of polymerized hemoglobin dose recovered and the content of reduced ($Fe^{2+}$) versus oxidized ($Fe^{3+}$) hemoglobin. Renal iron content was measured using a ferrozine-based colormetric assay. (**b**) Increased HO-1 immunoreactivity and non-heme iron deposition in renal proximal tubules 24 h after polymerized hemoglobin infusion. Non-heme iron was stained using the Perls method with DAB intensification. (**c**) HO-1 and ferritin upregulation following polymerized hemoglobin infusion. Kidneys were harvested from control animals and 4, 12, and 24 h post-infusion. Tissue extracts were prepared and analyzed by Western blot for HO-1 and L-ferritin. Representative immunoblots for HO-1 and L-ferritin are shown with each lane representing a different animal at each time point

constitute important sources of intracellular non-heme iron. Tissue non-heme iron visualization using the conventional Perls histochemical method has been vastly improved with the advent of various intensification methods (Meguro et al. 2007). Taken together, HO-1, iron accumulation and ferritin expression are potentially valuable markers of Hb exposure within tissue (Fig. 26.4).

### *26.5.2 End Products of Oxidative Damage*

4-hydroxy-2-nonenal (4-HNE) is, a major lipid peroxidation product of n-6 polyunsaturated fatty acid, contains a hydroxy group, conjugated C=C double bond, and a carbonyl group which renders this molecule highly reactive (Schaur 2003). The reaction of 4-HNE with proteins, and in particular, with the side chains of three main amino acids, cysteine, arginine, and histidine, can lead to loss of protein integrity and function. Measurement of 4-HNE modified protein adducts has become one of the most reliable indices of in vivo oxidative stress. Recent studies using commercialized antibodies for 4-HNE adducts have shown increased levels of 4-HNE adducts by immunohistochemistry and western blot in various tissues including kidney, brain and heart, following exchange transfusion with stroma-free Hb and polymerized Hbs (Boretti et al. 2009). Several analytical approaches using mass spectrometry, HPLC, and certain colorometric assays have also been developed to measure 4-HNE adducts with advantages and disadvantages associated with each method (Poli et al. 2008). Nuclear DNA oxidation produces 8-hydroxy-2′-deoxyguanosine (8-OHdG) which has been widely used as a biomarker for oxidative stress. Sensitive quantitative analysis of 8-OHdG is possible with high-performance liquid chromatography (HPLC) with electrochemical detection (EC), gas chromatography-mass spectrometry (GC–MS), and HPLC tandem mass spectrometry (Mateos and Bravo 2007). Commercially available antibodies for 8-OHdG have also been developed. Other oxidative reaction products such as oxidized low-density lipoprotein (OxLDL) and isoprostanes may be useful as predictive plasma markers for cardiovascular risk assessment (Tsimikas 2008).

### *26.5.3 Inflammation, Apoptosis and Necrosis*

Activated resident or circulating inflammatory cells accumulating at the endothelial lining act as an important source of ROS/RNS that could exacerbate the oxidative reactions of Hb, heme, and/or iron (Butt et al. 2011; Schaer et al. 2006). Within the CNS, brain microglia express ionized calcium binding adaptor molecule (iba-1) in response to inflammatory processes. Following drug-induced oxidation of Hb in a guinea pig model, brain microglia activation characterized by increased iba-1 expression was observed in conjunction with decreased neuronal

nuclei staining (Buehler et al. 2011). Astrocyte activation with enhanced glial fibrillary acidic protein (GFAP) expression was observed around HO-1 positive blood vessels in guinea pig brain following polymerized Hb infusion (Butt et al. 2011) (Fig. 26.2b). Endothelial upregulation of vascular cell adhesion molecule-1 (VCAM-1) was associated with subsequent pulmonary blood vessel wall thickening and remodeling in rats exposed to chronic low plasma concentrations of Hb (Buehler et al. 2012).

Abnormal or uncontrolled inflammation typically leads to cell death and subsequent tissue regenerative processes. Conventional toxicological methods are not generally amenable or designed to detect more *subtle* events such as apoptosis. Apoptotic changes can be difficult to capture as resolution can take place within hours. Unless studies are specifically tailored to monitor for these events clues into possible product-related toxicities may go completely unrecognized. Many methods are available for detecting apoptosis in cell culture, however, most of these approaches can not be applied in vivo especially in typical paraffin-embedded tissues (Barrett et al. 2001). Apoptotic nuclear changes such as DNA fragmentation are most commonly evaluated using the TUNEL assay. Over the years, the TUNEL assay has improved as far as implementation and interpretation, however, there are still potential problems associated with false positive findings and, thus, this approach is typically combined with other confirmatory assays such as measuring the markers of caspase activation, the specific regulators of the apoptotic cascade (Gown and Willingham 2002). Antibodies specific for the activated cleaved caspase forms as well as for caspase target proteins (e.g. PARP, lamin) are commercially available and applicable for routine immuno-based analyses.

## 26.6 Conclusion and Future Direction

The pre-clinical and Phase I evaluation of previous HBOCs did not effectively predict adverse events in later Phase clinical trials involving complex disease states (Silverman and Weiskopf 2009a). This may have been the result of two unforeseen issues; first, pre-clinical studies are conducted in normal animals and safety signals may be subtle and below the limits of detection for most GLP toxicology studies. Within the context of this chapter, several markers that indicate changes toward pathological processes in otherwise normal animal are discussed. It may be beneficial for future progress of the field to evaluate non-traditional toxicological markers to better HBOC candidate safety. Second, underlying disease states, such as diabetes or genetic risks that increase vascular dysfunction may have contributed to the observations of increased adverse events (Silverman and Weiskopf 2009a; Natanson et al. 2008). It may also be beneficial to the future progress of the field to consider evaluation of well validated animal models of vascular dysfunction. Critical to such models is the ability to define measurable toxicological endpoints that can distinguish underlying disease from additive

effects of HBOC administration. Future clinical study of HBOCs may require a more stringent and unique approach to pre-clinical development. The present chapter suggests novel ways to evaluate pre-clinical safety of HBOCs.

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# Chapter 27
# The Hemoglobin-Based Oxygen Carrier, HBOC-201, as a Resuscitation Fluid for Traumatic Hemorrhagic Shock: The Naval Medical Research Center Experience

**Charles Auker, Paula Moon-Massat, Anke Scultetus, Richard McCarron and Daniel Freilich**

## 27.1 Background

In response to the terrorist attacks in the United States on September 11, 2001 and the subsequent advent of conflicts in Afghanistan and Iraq, the Naval Medical Research Center (NMRC, Silver Spring, MD) initiated a search for an oxygen-carrying resuscitation fluid that could be used for combat trauma and in situations with wide-spread civilian casualties. The ability to supply blood to mass casualties or far-forward combat trauma victims in austere environments with long evacuation times is wrought with logistical complications. Exsanguinating hemorrhage accounts for the preponderance (60 %) of civilian and potentially salvageable combat deaths (Butler et al. 2007). In Operation Iraqi Freedom (Iraq war) and Operation Enduring Freedom (Afghanistan war), over 80 % of potentially survivable fatalities were due to hemorrhage, more than half of which occurred prior to hospital arrival (Kelly et al. 2008). For casualties in severe hemorrhagic shock, blood transfusions can be life-saving pending definitive surgical stabilization, but deployment of blood transfusion capability is logistically costly and complex. Blood must be refrigerated, typed, cross-matched to the recipient, administered through specialized filters and by specialty-trained personnel and has a relatively short shelf-life before it expires. It is rarely available in rural civilian pre-hospital

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settings or far-forward military medical units where lives might be saved with effective alternative therapy.

The long-range goal of NMRC's program has been to develop a multifunctional blood substitute that has oxygen-carrying, hemostatic, and anti-inflammatory capabilities. A shorter-term goal is to gain approval for an oxygen-carrying resuscitation fluid for the indication of severe hemorrhage when blood is not immediately available. A fluid with low logistic burden (e.g., weight, volume, thermal and temporal storage limitations) is necessary to meet military requirements.

In 2003 NMRC created an advisory committee of trauma experts to identify and recommend an oxygen therapeutic (OT) for further development and ultimately for use in a clinical trial for the stated indication. Any type of asanguinous OT was considered, particularly hemoglobin-based oxygen carriers (HBOCs) or perfluorocarbons (PFCs). The committee's analysis and selection was based on a number of militarily-relevant factors, including universal compatibility with all blood types, long-term stability without refrigeration, low volume, light weight, a readily available source for commercial production with demonstrated manufacturing capability, and agreement by the manufacturer to permit autonomous government sponsorship of a clinical trial. With these criteria and after evaluating the most developed OTs at the time, the advisory committee recommended HBOC-201 (Hemopure®, Biopure Corporation [now OPK Biotech], Cambridge, MA) for further evaluation.

In 2004, NMRC submitted to the FDA a proposal for a trauma trial. The initial proposal was for a multi-step Phase 2 leading to Phase 3 multi-center trial for the indication of traumatic hemorrhagic shock when access to blood was not readily available, targeting civilian trauma victims under an exception from informed consent (EIC). The outcome of early FDA interactions was a request by FDA reviewers for several additional pre-clinical efficacy studies in specific, clinically relevant, scenarios with severe hemorrhage. FDA was particularly interested in a comprehensive understanding of efficacy and safety of HBOC-201 in situations with prolonged delay to definitive care and in polytrauma situations that included traumatic brain injury (TBI). NMRC therefore embarked on a systematic evaluation of HBOC-201 using invasively-instrumented swine models of controlled hemorrhage alone, uncontrolled hemorrhage alone, and polytrauma (controlled or uncontrolled hemorrhage with TBI). In addition to its own studies, NMRC supported and collaborated with investigators at other institutions. This chapter summarizes the NMRC experience, to date, in pursuing its goal of a multifunctional blood substitute for trauma resuscitation.

## 27.2 HBOC Efficacy Studies

NMRC designed its intramural pre-clinical swine models to mimic the course of events relevant to combat trauma in an austere battlefield environment or civilian setting where delayed hospital arrival was anticipated. Efficacy endpoints such as

survival, hemodynamics, and primary or secondary indicators of tissue perfusion or oxygen delivery were evaluated. These trauma models were necessarily divergent from a true combat trauma event. The swine were anesthetized and otherwise healthy (without apparent concurrent systemic diseases). Hemorrhage was either controlled via catheter withdrawal with or without a muscle crush injury or uncontrolled via a standardized liver laceration. TBI, when included, was imparted only by fluid percussion (FP) rather than a combination of penetrating or concussive blast injuries. Otherwise, all attempts were made to treat the swine as a combat or civilian trauma victim with a varying delay to definitive care (Table 27.1; Fig. 27.1). Swine were randomized to different resuscitation treatments where the control groups were the colloid hetastarch (HTS) or the crystalloid lactated Ringers (LR) plus a no-resuscitation group (NON). The rationale for these choices were that HTS mimics the military's in-field resuscitation fluid of choice, LR is the civilian paramedic's resuscitation fluid of choice and the NON group not only mimics the "scoop and run" philosophy of urban pre-hospital trauma care but also provides an absolute negative control. Of note, blood was not used as a control fluid because the hypothesis was to evaluate HBOC-201 versus the pre-hospital standard-of-care, not the theoretical "best treatment" option. Furthermore, consistent with NMRC's clinical trial proposal, groups were treated differently only during the pre-hospital phase; once hospital arrival was simulated, all animals were eligible (per protocol) to receive equivalent in-hospital standard-of-care, including additional fluid or blood transfusions (no further HBOC-201) and other supportive measures as determined by the injury type. Importantly, therefore, the results of these studies showed group differences in a realistic clinical setting but were equivalently controlled.

NMRC performed three controlled hemorrhage and three uncontrolled hemorrhage studies of escalating severity (Table 27.1). The least severe was a controlled hemorrhage study with removal of 40 % of the estimated blood volume (EBV) and a 4 h delay to simulated hospital arrival (Philbin et al. 2005). A more severe controlled hemorrhage model, with 55 % EBV hemorrhage, was used in two other studies (Rice et al. 2006b; Philbin et al. 2007), the first of which used a 4 h delay to simulated hospital arrival (Rice et al. 2006b), while the second used a prolonged 24 h delay (Philbin et al. 2007). In all cases of controlled hemorrhage, the total observation duration was 72 h.

In the less severe (40 %) model, survival of the three groups (HBOC-201, HTS, NON) was statistically equivalent, but with a possible trend favoring HBOC-201 resuscitation. HBOC-201 was at least as efficacious as HTS in restoring hemodynamics, improving local tissue oxygenation, and decreasing fluid requirements. In both of the more severe (55 %) studies, HBOC-201 and HTS had statistically significant survival advantages over no resuscitation (NON); in the 4 h delay study (Rice et al. 2006b) HBOC-201 exhibited a non-significant trend in survival advantage over HTS. In all three studies, HBOC-201 improved hemodynamics, transcutaneous tissue oxygen tension, and transfusion avoidance compared to HTS.

NMRC then advanced to three studies using more severe uncontrolled hemorrhage models that more closely mimic clinical trauma situations. The first study

**Table 27.1** HBOC-201 efficacy studies

| Injury model | Species | Transport time | Post-injury duration (h) | HBOC survival | Control survival, fluid type | *P* value | Study location | Reference |
|---|---|---|---|---|---|---|---|---|
| 40 % EBV controlled hemorrhage | Swine | 4 h | 72 | 8/8 (100 %) | 7/8 (88 %) HTS, 5/8 (63 %) NON | NSD versus both HTS and NON | NMRC | Philbin et al. (2005) |
| 55 % EBV controlled hemorrhage | Swine | 4 h | 72 | 8/8 (100 %) | 6/8 (75 %) HTS, 2/8 (25 %) NON | NSD versus HTS | NMRC | Rice et al. (2006b) |
| 55 % EBV controlled hemorrhage | Swine | 24 h | 72 | 7/8 (88 %) | 7/8 (88 %) HTS, 2/8 (25 %) NON | NSD versus HTS; <0.05 versus NON | NMRC | Philbin et al. (2007) |
| Severe, uncontrolled hemorrhage | Swine | 4 h | 72 | 7/8 (88 %) | 1/8 (13 %) HTS, 1/8 (13 %) NON | <0.01 versus both HTS and NON | NMRC | Gurney et al. (2004) |
| Severe, uncontrolled hemorrhage + FP-TBI | Swine | 75 min | 6 | 8/13 (62 %) | 1/11 (9 %) LR, 1/8 (13 %) NON | < 0.05 versus both LR and NON | NMRC | Stern et al. (2009) |
| Severe, uncontrolled hemorrhage + FP-TBI | Swine | 30 min | 6 | 5/9 (56 %) | 4/9 (44 %) LR, 4/8 (50 %) NON | NSD versus both LR and NON | NMRC | Teranishi et al. (2012) |

*EBV* estimated blood volume, based on 65 ml/kg, *FP-TBI* fluid percussion traumatic brain injury, *HTS* hetastarch, *LR* lactated ringers, *NMRC* Naval Medical Research Center, *NON* no resuscitation, *NSD* no significant difference, *OT* oxygen therapeutic

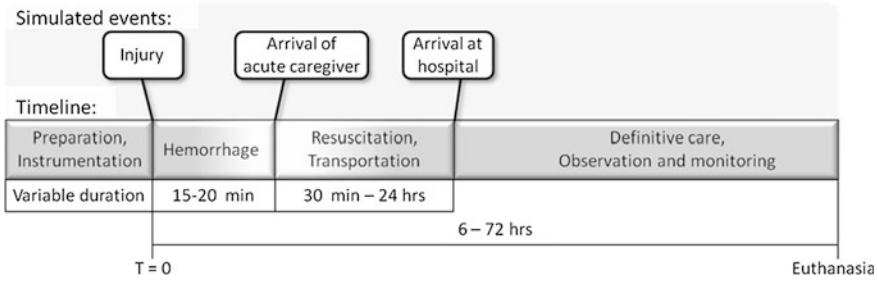


**Fig. 27.1** NMRC's standard swine trauma and resuscitation study timeline. See References for specific details of individual studies

was similar to the earlier controlled hemorrhage models (simulated evacuation delay was 4 h and the total observation period was 72 h) except that the injury was a standardized liver laceration/crush and hemorrhage was uncontrolled (Gurney et al. 2004). In comparison to the controlled hemorrhage studies, in this uncontrolled hemorrhage study HBOC-201 significantly improved 72 h survival compared to the control fluids (Table 27.1). HBOC-201 also improved hemodynamics, transcutaneous tissue oxygen tension, and transfusion avoidance compared to HTS.

The two other uncontrolled hemorrhage studies were extreme injury models that included concurrent FP-TBI and while similar to the previous NMRC studies, they had a few crucial differences (Stern et al. 2009; Teranishi et al. 2012). First, at the behest of the FDA, delay to definitive treatment was shortened to be more consistent with civilian scenarios and closer to the proposed clinical civilian trauma trial. To that end, two simulated transport times (75 and 30 min) were studied. Secondly, because of the limitations imposed by the cerebral instrumentation, the observation and monitoring period was reduced to 6 h post-injury. Finally, the control resuscitation fluid was LR, not HTS. The results of these studies indicated that fluid and blood transfusion requirements, systemic and cerebrovascular physiologic parameters, and indices of systemic and cerebral tissue perfusion were significantly improved with HBOC-201 for both the 75 and 30 min pre-hospital transport times (Stern et al. 2009; Teranishi et al. 2012). HBOC-201 significantly improved 6 h survival only for the 75 min pre-hospital delay (Table 27.1). It was concluded that uncontrolled hemorrhage with concomitant FP-TBI could be adequately managed with HBOC-201 and that HBOC-201 appeared to confer a benefit over the current standard-of-care for the longer evacuation time (up to the 75 min evaluated).

## 27.3 HBOC Basic Science and Safety Studies

In addition to investigating efficacy endpoints, NMRC examined safety data (e.g., hematologic, immunologic, and histopathologic) from the foregoing experiments (Table 27.2). While limited by various aspects of study design (which were

**Table 27.2** Basic science and safety studies using HBOC-201

| Species | Focus of study | Study location | Reference |
|---|---|---|---|
| Swine | Coagulation in controlled hemorrhage and HBOC resuscitation | NMRC | Arnaud et al. (2005) |
| Swine | Coagulation in severe uncontrolled hemorrhage and HBOC resuscitation | NMRC | Arnaud et al. (2006) |
| Swine | Immune parameters in controlled hemorrhage and HBOC resuscitation | NMRC | Dong et al. (2006) |
| Swine | Vasoactivity of HBOC resuscitation in hemorrhage with and without TBI | NMRC | Rice et al. (2006a) |
| Swine | Histopathology and organ function in models of hemorrhage and HBOC resuscitation | NMRC | Johnson et al. (2006) |
| Swine | Hematology patterns in severe controlled hemorrhage and HBOC resuscitation | NMRC | Arnaud et al. (2007) |
| Swine | Immune responses in severe hemorrhage and HBOC resuscitation | NMRC | Hall et al. (2007) |
| Swine | Amylase and lipase in hemorrhage and HBOC resuscitation | NMRC | Arnaud et al. (2011a) |

powered for survival and hemodynamic analysis), results of these ancillary analyses were remarkable primarily in revealing no consistent, unequivocal, or clinically-relevant adverse effects of HBOC-201 on coagulation or immune status. Chemical markers of organ function were mostly unremarkable, but there was evidence of elevated lipase and higher peak creatinine kinase (CK) activity with HBOC-201 resuscitation. Myocardial and pulmonary histopathology results were generally similar between HBOC-201 and comparator groups, but there was some indication of adverse hepatobiliary and renal papillary histopathological effects.

Collectively, the results of the HBOC-201 pre-clinical studies (Tables 27.1, 27.2) demonstrated that the superiority of survival with HBOC-201 occurred when the severity of the insult was greatest or when the delay to definitive treatment (including surgery or blood transfusions) was prolonged. Standard resuscitation appeared adequate in the mild to moderate injury models, in which the bleeding had stopped, volume restoration was more important than increasing oxygen carrying capacity, and compensatory physiological responses were adequate for the injury. However, HBOC-201 provided demonstrably improved survival benefit in the more severe injuries or with prolonged transport times, those situations with the most devastatingly poor outcomes. This is a crucial point in determining the risk to benefit relationship of HBOC resuscitation, a calculus that would be different for mild- moderate hemorrhage versus severe hemorrhagic shock. Figure 27.2 illustrates this point by summarizing survival data from NMRC's and other pre-clinical hemorrhage studies spanning a range of injury severities.

It should be noted that the NMRC studies described above employed low-pressure, solid organ injury as the injury for uncontrolled hemorrhage. In a more recent study performed at the University of Washington (White et al. 2013) a high-pressure, aortic tear injury (with and without TBI) polytrauma swine model was

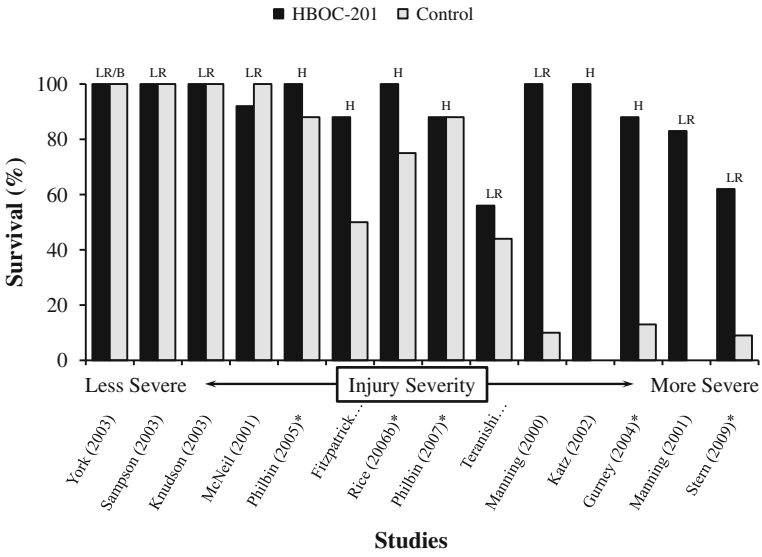


**Fig. 27.2** Dependency of HBOC-201 survival efficacy on the severity of hemorrhage. Severity of hemorrhage progresses from controlled moderate hemorrhage models on the *left*, to uncontrolled severe hemorrhage models on the *right*. Note that the Teranishi et al. (2012) and Stern et al. (2009) studies differ only in their delay to definitive care (Teranishi: 30 min, Stern: 75 min). For brevity, only first authors are used to designate studies. *Asterisks* (*) denote studies performed at NMRC. Control fluids varied and are indicated for each study above each set of bars: LR = Lactated Ringer, B = Blood, H = Hetastarch

studied. In this study, HBOC-201 resuscitation decreased survival time compared to LR resuscitation. Whether or not similar adverse results would be seen in a model of intermediate sized vessel injury (e.g., iliac or femoral vascular injuries) is unclear. Results from an earlier NMRC swine study of uncontrolled hemorrhage from an iliac artery injury suggest that blood loss and survival are equivalent to HTS or normal saline (NS) when using hypotensive resuscitation with HBOC-201 (Bowyer 2005). However, this study could not be considered definitive due to numerous flaws and the fact that the injury was not solely an arterial injury. Thus further investigation is required to elucidate whether or not there are differences between HBOC-201's effects in large, medium or small arterial injuries.

Thus, NMRC-sponsored preclinical studies suggest that the clinical utility of HBOC-201 appears to depend on hemorrhage severity and injury type: high predicted efficacy in severe hemorrhage due to solid organ injury without concomitant TBI (Fig. 27.2), and low efficacy in uncontrolled hemorrhage due to aortic injury with or without concomitant TBI.

NMRC also explored adding a hemostatic agent, recombinant factor VIIa (rfVIIa), to HBOC-201 in pursuit of its long-range goal of a multifunctional blood substitute. In two studies, rFVIIa was added to HBOC-201 resuscitation using the NRMC swine uncontrolled hemorrhage model (Table 27.3). Results from the first

**Table 27.3** Summary of NMRC studies exploring addition of hemostatic agent, rFVIIa, to HBOC-201 for resuscitation of uncontrolled hemorrhagic shock

| OT | Injury model | Species | Transport time (h) | Post-injury duration (h) | HBOC survival | HBOC + rfVIIa survival | Control survival, fluid type | P-value | Study location | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| HBOC-201 ± 90, 180, or 360 μg/kg rFVIIa | Severe, uncontrolled hemorrhage | Swine | 4 | 72 | 2/8 (25 %) | 14/24 (70.8 %) rfVIIa combined | None | NSD for all rFVIIa doses; < 0.05 versus HBOC-90 μg/kg rFVIIa | NMRC | Scultetus et al. (2011) |
| HBOC-201 ± 90 μg/kg rFVIIa | Severe, uncontrolled hemorrhage | Swine | 1 | 72 | 14/22 (64 %) | 15/19 (79 %) | 0/4 (0 %) NON | NSD between OT groups | NMRC | Haque et al. (2012) |

*NSD* no significant difference, *NON* no resuscitation, *OT* oxygen therapeutic

study indicated that low dose (90 μg/kg) but not high dose (360 μg/kg) rFVIIa improved cardiac output and anaerobic metabolism when added to HBOC-201 resuscitation. There were trends toward improved hemorrhage volume and survival, expected coagulation effects and minimal immune activation (Scultetus et al. 2011; Malkevich et al. 2008; Arnaud et al. 2008). Despite using a larger number of animals in the second study, a dose of 90 ì g/kg rFVIIa did not show any statistical improvement in blood loss, physiologic parameters or, more importantly, survival when it was added to HBOC-201 in a swine model of severe hemorrhage (Haque et al. 2012). However, optimization of the components, their mixing ratio, and the timing and duration of administration might identify a beneficial regimen but would require additional research.

## 27.4 VA-OT Program

While the foregoing NMRC-sponsored studies were designed as primarily efficacy studies, they also provided information related to potential safety concerns expressed by the FDA and others (Natanson et al. 2008). One of the most prominent concerns was vasoconstriction, considered to be a characteristic class effect of HBOCs. It was postulated that vasoconstriction might be the underlying mechanism for the imbalance in ischemic vascular adverse events such as myocardial infarction or stroke observed in previous HBOC clinical trials (Natanson et al. 2008). Another concern was that HBOC-induced blood pressure elevations might be misinterpreted by first-responders in a resuscitation scenario as an indication of adequate fluid replacement, resulting in under-resuscitation, inadequate tissue perfusion, and ultimately a poorer outcome than the current standard-of-care. Finally, there was concern that any extreme or rapid elevations in blood pressures might increase the volume of blood loss and/or the risk of re-bleeding, thereby worsening outcome.

The majority of NMRC's hemorrhagic shock studies revealed higher mean arterial pressures in HBOC-201 resuscitated swine than in the control groups. However, the study endpoints also suggested that elevations of blood pressure were not harmful and actually might be beneficial in hemorrhage scenarios. Survival and physiologic indicators of tissue oxygenation were equivalent to, or improved over, comparator groups, and fluid requirements following simulated hospital arrival were lower. As previously noted, in the most severe hemorrhage models, survival was significantly improved by HBOC-201. These findings are supported by independent preclinical data suggesting that vasoconstricting drugs, when administered with volume repletion and efforts to rapidly control hemorrhage, may be beneficial in trauma (Feinstein et al. 2005a, b; Sanui et al. 2006).

Nonetheless, to enable progress with an HBOC trauma IND, preclinical data was required that evaluated efficacy and safety results of an OT with little or no inherent vasoconstricting properties. In pursuit of this goal NMRC structured a second OT-focused program seeking a non-vasoconstrictive formulation (termed "VA-OT" for vasoconstricting-attenuated OT).

The NMRC VA-OT program is an on-going, extensive, international collaborative effort that uses a tiered methodology to identify a military-relevant VA-OT (Fig. 27.3). The overall strategy is to screen candidate solutions using in vitro preparations and simple in vivo models; then advancing the most promising candidate(s) to more complex swine injury models. Stage 1 is a dual-pronged approach that attempts to attenuate HBOC-201's vascular reactivity by either chemically modifying the formulation (termed "intrinsic" efforts) or pharmacologically mitigating its vasoconstricting effects (termed "extrinsic" efforts). A study matrix (Tables 27.4, 27.5) illustrates the in vitro and in vivo preclinical models for both intrinsic and extrinsic tactics, respectively. In Stage 2, the search for a VA-OT was widened beyond HBOC-201 to include not only other HBOCs but also perfluorocarbons (PFC). The Stage 2 study matrix, similar to Stage 1, first determines the vasoactive characteristics of each OT before advancing to complex injury models. These next-generation compounds still must meet the original criteria outlined by the original NMRC trauma advisory committee to be attractive for military use. Since all Stage 2 studies are currently ongoing, the remainder of this chapter provides an overview of Stage 1 VA-OT investigations only. Study summaries are based on either published literature or final study reports (unpublished data) submitted to NMRC.

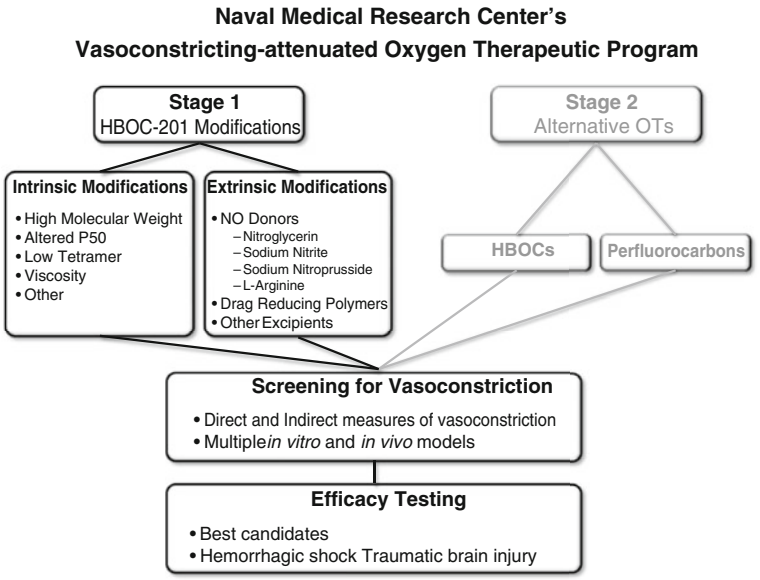


**Fig. 27.3** Schematic of NMRC's vasoconstricting-attenuated oxygen therapeutic (VA-OT) Program

**Table 27.4** Study matrix of intrinsic approach to VA-HBOC-201

| Study model | Location(s) of study (ies) | HBOC-201 P50 34-46 mm Hg viscosity ~3 cP[a] | Modified HBOC-201 designation | | | | |
|---|---|---|---|---|---|---|---|
| | | | HBOC-201.LT.DL | HBOC-205.LT.LL.MW400 | HBOC-205.LT.LL.MW600 | HBOC-206.LL.LT.MP50 P50 ~17 mm Hg Viscosity ~4 cP[a] | HBOC-206.LL.LT.LP50A P50 ~17 mm Hg Viscosity ~12 cP[a] |
| In vitro models | | | | | | | |
| Vascular strips | Brown University | × | × | | × | | |
| Aortic rings | UAB | × | × | | × | | |
| In vivo models | | | | | | | |
| Rodent toploaad | Erasmus, VCU | × | × | × | × | × | × |
| Rodent hemorrhage | UAB | × | × | × | × | | |
| Swine injury | NMRC, Univ. Miami | × | × | | | | |

*Erasmus* Erasmus Medical Center, Rotterdam, The Netherlands, *NMRC* Naval Medical Research Center, *UAB* University of Alabama at Birmingham, *VCU* Virginia Commonwealth University

[a] Viscosity measurements taken at room temperature

**Table 27.5** Study matrix for extrinsic approaches to VA-HBOC-201

| Models | Location(s) of study/ies | HBOC-201 | HBOC plus | | | |
|---|---|---|---|---|---|---|
| | | | NTG | SNP | Nitrite | Other |
| In vitro | | | | | | |
| Vascular strips | Brown University | × | × | × | × | |
| Aortic rings | UAB | × | × | × | × | |
| Fluid mechanic | University of Pittsburgh | × | | | | × (DRP) |
| In vivo | | | | | | |
| Rodent, topload | Erasmus, NIH, VCU | × | × | × | × | × (Sildenafil)[a] |
| Rodent, hemorrhage | UAB | × | × | × | × | |
| Swine, topload, awake | Erasmus | × | × | × | × | × (Adenosine) |
| Swine, injury models | NMRC, UNC, University of Michigan University of Washington | × | × | × | × | × (L-Arginine) |

*DRP* drag reducing polymer, *Erasmus* Erasmus Medical Center, Rotterdam, The Netherlands, *NIH* National Institutes of Health, *NMRC* Naval Medical Research Center, *UAB* University of Alabama at Alabama, *UNC* University of North Carolina at Chapel Hill, *VCU* Virginia Commonwealth University

[a] Sildenafil was evaluated with HBOC-301 rather than HBOC-201

### *27.4.1 Stage 1 VA-HBOC-201 Intrinsic Approach*

Stage 1 and 2 studies were performed in collaboration with Biopure Corporation (currently OPK Biotech, Cambridge, MA), the manufacturer of HBOC-201. In particular, Biopure manufactured a number of small experimental batches of modified HBOC-201, thereby allowing NMRC to evaluate some of the hypotheses for mitigating HBOC-201-induced vasoconstriction.

**Molecular weight (MW)**. There are at least two ways in which changes in MW distribution might affect the vasoconstricting tendency of HBOC-201. Firstly, reducing the tetramer content had previously been shown to reduce the vasoactivity in earlier generation HBOCs (e.g., DCHLb). In addition, HBOC-201 with $<3$ % tetramer appeared to be less vasoconstrictive than Biopure's similar veterinary product (HBOC-301) with 35 % tetramer (although a direct head to head comparison was not done). Secondly, the larger the HBOC molecules, the more likely they are to be retained within the vasculature and, unable to extravasate, the less likely to scavenge NO which is most active in the perivascular space. Thus, NMRC hypothesized that these two characteristics had not been optimized for HBOC-201. Therefore, the amount of tetramer was decreased from $<3$ % in HBOC-201 to $\leq 0.4$ % (this compound was identified as HBOC-201.LT.DL or HBOC-201.LT.LL) and, by keeping this lower tetramer content and further polymerizing the compound, the mean MW was increased to 400 or 600kD (these compounds were identified as HBOC.LT.LL.MW400 or HBOC.LT.LL.MW600, respectively). These target MW modifications were selected based on the likelihood of future manufacturing

capabilities (i.e., manufacturing costs, etc.). To conserve resources, the HBOC.LT.LL.MW600 was often screened without concurrent testing of HBOC.LT.LL.MW400 as it seemed logical to assume that if no differences were observed compared to HBOC-201, then there was no added value in evaluating the intermediary compound. However, if differences were observed, then the intermediate formulation would be tested.

**P50 and viscosity**. To test whether changing the P50 might affect HBOC-201's vasoconstricting effects, it was reduced from ~40 to 17–18 mm Hg. One of these new lower P50 compounds (HBOC-206.LL.LT.MP50) had a similar viscosity to blood (~4 cP) while the other (HBOC-206.LL.LT.LP50A) was formulated with a higher viscosity (~12 cP). Testing these compounds in a small pilot study (VCU, Dr. R. Pittman, PI) allowed a cursory assessment as to whether or not P50 or viscosity played a role in HBOC-201 vasoactivity.

**Intrinsic approach results**. None of the "intrinsic approach" in vitro or in vivo studies showed consistent evidence of clinically relevant reductions in HBOC-201-induced vasoconstriction after chemically modifying its structure by reducing the tetramer content, increasing the MW (or both), lowering the P50 or increasing the viscosity (Tables 27.6, 27.7 for specific studies). The individual studies provided some subtle differences in the hemodynamic responses to the various HBOC compounds but, most importantly, the vasoconstrictive effects were similar to those observed with HBOC-201 and none of these modified compounds were evaluated further (Tables 27.6, 27.7). Nonetheless, it should be noted exploration of modifications outside the range tested, such as an extremely low P50 (<10 mm Hg), may have yielded changes in vasoactivity.

### *27.4.2 Stage 1 VA-HBOC-201 Extrinsic Approach*

In parallel to the intrinsic approach, a number of compounds, with widely varying mechanisms of action, were administered concurrently with HBOC-201 (extrinsic approach) in an attempt to pharmacologically mitigate its vasoconstricting effects. Due to limited resources, complete dose-response studies (to select the most favorable doses) were not performed for any of the concurrent drugs (often only one or two doses were evaluated), nor were studies done to optimize the timing of adjuvant drug administration (i.e., before vs. during the HBOC administration). Thus, NMRC considers this data as preliminary and results do not definitively establish whether or not this approach to achieving a VA-HBOC-201 was successful.

**NO donor drugs**. Based on the concept that most of the HBOC-induced vasoconstriction was due to NO scavenging, the most promising class of drugs was the NO donors (or an NO-precursor) because these drugs would directly replenish the NO pool. As a proof of concept, earlier work had shown with the first generation HBOC DCLHb (which had more potent vasoconstricting properties) that its vasoconstrictive effects could be reduced by NO donors (Bilello et al. 1994; Rioux et al. 1995; Erhart et al. 2000). Inhaled NO (iNO) also was shown to reverse

**Table 27.6** Intrinsic and extrinsic VA-HBOC-201 efficacy (survival) studies

| OT versus HBOC-201 | Model | Species | Transport time | Post-injury duration | HBOC-201 survival | VA-HBOC survival | Control survival | P-value | Study location (PI) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| HBOC-201.LT.DL | Blunt trauma to head (TBI), chest, and bilateral femur fxs (ISS: 27–41) | Swine | 45 min | 8.8 h | 14/14 (100 %) | NSD[b] | 10/14 (71 %) NS | NSD | University of Miami (Proctor) | Dudkiewicz et al. (2008) |
| HBOC-201.LT.DL; HBOC-301 | Controlled hemorrhage (55 % EBV) | Swine | 74 min | 4 h | 9/9 (100 %) | 8/9 (89 %) | N/A | NSD | NMRC | Rice et al. (2008) |
| HBOC-201 + 0.8 μg/kg/min SNP | Controlled hemorrhage (55 % EBV) | Swine | 60 min | 2 h | 4/4 (100 %) | 7/7 (100 %) | 3/4 (75 %) HTS | NSD | NMRC | Arnaud et al. (2012) |
| HBOC-201 + 500 mg/kg L-Arginine | Controlled hemorrhage (55 % EBV) | Swine | 90 min | 4 h | 8/8 (100 %) | 8/8 (100 %) | 8/8 (100 %) NS; 3/7 (43 %) NON | NSD versus NS; <0.01 either OT versus NON | University of Michigan (Stern) | Stern (2010a), Unpublished |
| HBOC-201 + 0.54, 1.08 or 1.62 μmol/kg Na Nitrite | Controlled hemorrhage (55 % EBV) | Swine | 60 min | 2 h | 6/6 (100 %) | 18/18 (100 %)[c] | 4/5 (80 %) HTS | NSD | NMRC | Arnaud et al. (2011b) |
| HBOC-201 + 0.54 or 0.08 μmol/kg/min Na Nitrite | Uncontrolled hemorrhage (80 % EBV) | Swine | 75 min | 72 h | 3/5 (60 %) | 3/8[a] (38 %) | 1/5 (20 %) HTS | N/A[a] | NMRC | Moon-Massat et al. (2012) |

(continued)

**Table 27.6** (continued)

| OT versus HBOC-201 | Model | Species | Transport time | Post-injury duration | HBOC-201 survival | VA-HBOC survival | Control survival | P-value | Study location (PI) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| HBOC-201 + 5 μmol/kg/min NTG | Uncontrolled hemorrhage | Swine | 75 min | 72 h | 3/5 (60 %) | 5/5 (100 %) | 3/6 (50 %) HTS | NSD | NMRC | McCarron (2012), Unpublished |
| HBOC-201 + 2.5 ug/kg/min NTG | Uncontrolled hemorrhage (liver) + FP-TBI | Swine | 75 min | 8 h | 4/6 (67 %) | 1/6 (17 %) with 2.5 μg/kg/min<br>0 % with 5 μg/kg/min | HTS | <0.05 | NMRC | Scultetus et al. (2011) |
| HBOC-201 + 2.5 ug/kg/min NTG | Uncontrolled hemorrhage (arterial) + FP-TBI | Swine | 105 min | 6 h | 0/5 (0 %) | 2/5 (40 %) | 3/5 (60 %) LR | <0.05 | University of Washington (Stern) | Stern (2010b), Unpublished |

Note that studies often had multiple control groups (such as NO donor alone, NO donor + CNTL fluid and/or NON). Not all control groups are listed here, only the primary control fluid (standard of care), please see references for further details

*ISS* injury severity score, *NS* normal saline, *FP-TBI* fluid percussion traumatic brain injury, *HTS* hetastarch, *LR* lactated ringers, *fxs* fractures, *NSD* no significant difference, *NTG* nitroglycerine, *SNP* sodium nitroprusside

[a] Due to humane euthanasia of some animals, spontaneous mortality could not be calculated or compared

[b] LT-HBOC versus HBOC-201 NSD, so groups combined and compared to NS control group

[c] Differences among three nitrite dose groups NSD, so all groups combined

EBV was calculated as 65 % of body weight

**Table 27.7** Intrinsic and extrinsic VA-HBOC-201 basic science and safety studies

| OT | Species | Study focus | Study location (PI) | Reference |
|---|---|---|---|---|
| HBOC-201, LT-HBOC-201 | Swine | Immune effects in severe controlled hemorrhage | NMRC | VanderMolen et al. (2007) |
| HBOC-201 + NO donors; other HBOC modifications | In vitro | Bovine isolated vascular rings | Brown Univ. (Hai) | Fonseca et al. (2010); Hai (2009) |
| HBOC-201 + NO donors; other HBOC modifications | Mice | Hemodynamic effects in anesthetized normovolemic mice | Erasmus (Duncker) | Duncker (2009a) |
| HBOC-201 + NO donors; other HBOC modifications | Swine | Hemodynamic and myocardial effects in awake, exercising swine | Erasmus (Duncker) | Duncker (2009b) |
| HBOC-201 + NO donors; other HBOC modifications | In vitro; Mice | Isolated aortic rings; Hemodynamic effects in normovolemic mice or after controlled hemorrhage | UAB (Kerby) | Rodriguez et al. (2009); Kerby (2009) |
| HBOC-201 + excipient | In vitro | Potential applications of drag-reducing polymers with HBOCs | Univ. of Pittsburgh (Kameneva) | Kameneva (2009), Unpublished |
| HBOC-301 + sodium nitrite or sildenafil | In vitro and rats | Assayed nitrite reductase activity of HBOC; Hemodynamic effects in normovolemic rats | NIH, Univ. of Pittsburgh (Gladwin) | Gladwin (2011), Unpublished |
| HBOC-201, other modifications | Rats | Direct measures of skeletal muscle or mesenteric vessel vasoactivity and tissue oxygenation | VCU (Pittman) | Unpublished data |
| HBOC-201 + 10, 20, or 40 ug/kg/min NTG | Swine | Controlled hemorrhage (55 % EBV) | UNC (Manning) | Katz et al. (2010); Manning (2009) |
| HBOC-201 + excipients | Rats | Direct measures of cerebral vessel vasoactivity | NMRC | Ongoing |

*DRP* drag reducing polymers, *Erasmus* Erasmus Medical Center, Rotterdam, The Netherlands, *NIH* National Institutes of Health, *NMRC* Naval Medical Research Center, *UAB* University of Alabama at Alabama, *UNC* University of North Carolina at Chapel Hill, *VCU* Virginia Commonwealth University

DCLHb-mediated pulmonary vasoconstriction (Sefton et al. 1999). This drug class was the one most extensively investigated by NMRC because of this earlier data and also because a number of NO donors were already FDA-approved and in clinical use as vasodilators (e.g., sodium nitrite for cyanide toxicity, sodium nitroprusside for malignant hypertension, and nitroglycerine for acute coronary syndromes). Thus background information on the safety and efficacy of the individual NO donors was available and a more rapid path to approval was conceivable. Four NO donor drugs were tested in NMRC-sponsored studies: L-arginine, sodium nitrite, nitroglycerin, and sodium nitroprusside.

**Other drugs**. The rationale for evaluating non-NO donor drugs was two-fold. Firstly, there is data to suggest that NO scavenging is not the only mechanism underlying HBOC-induced vasoconstriction (Fonseca et al. 2010). Secondly, one could argue that any mechanism mitigating HBOC vasoconstriction might be acceptable and restricting studies to directly antagonizing its actions were unnecessarily limiting. Based on these considerations two additional agents, adenosine (a purine with known vasodilatory effects) and hyaluronic acid (a drag-reducing polymer with potentially beneficial rheological effects) were evaluated in small screening studies.

## 27.5 Extrinsic Approach Results

Screening studies using in vitro isolated vessels (Rodriguez et al. 2009; Fonseca et al. 2010) and both normovolemic and hemorrhaged mice (Rodriguez et al. 2009; Duncker 2009a, Unpublished) supported the use of NO donors to blunt HBOC-201-mediated vasopressor effects. NMRC continued investigations of these compounds in swine models where it was then concluded that sodium nitroprusside and nitroglycerine remained the most viable options and L-arginine and sodium nitrite were eliminated from further studies.

*L-Arginine*. L-Arginine, an NO precursor, failed to blunt the HBOC-201-associated vasoconstrictive response in a swine controlled hemorrhage model, despite using doses up to 500 mg/kg IV (Stern 2010a, Unpublished). As higher doses of L-arginine would have been logistically difficult to administer, it was eliminated from further consideration.

*Sodium nitrite*. Sodium nitrite also was removed from further study by NMRC despite effectively mitigating HBOC-201-induced vasoconstriction (Moon-Massat et al. 2010; Arnaud et al. 2011b). In the 72 h survival, uncontrolled hemorrhage study in swine, the first concurrent infusion of sodium nitrite was able to attenuate, in a dose-dependent fashion, the pulmonary and systemic blood pressure increases associated with HBOC-201 infusions without apparent clinical or laboratory adverse effects. However, the beneficial hemodynamic effects were transient; repeated infusions were not as effective regardless of the dose of sodium nitrite, and by the third infusion, sodium nitrite was ineffective in reducing HBOC-associated vasoactivity. While variations in the repeat dosing regimen could be explored

further, unexpected adverse clinical side-effects necessitating early euthanasia, and the pathology findings from these animals, suggested the drug combination might present safety issues (Moon-Massat et al. 2010). Necropsy revealed a correlative excess of pulmonary microthrombi and hemorrhages in animals receiving both sodium nitrite and HBOC-201 although the underlying mechanism was not identified. Similar pathology or clinical signs were not observed in either the screening mouse studies or the initial swine study of controlled hemorrhage (Arnaud et al. 2011a, b); the latter of which used an identical treatment protocol. Neither of those studies permitted recovery of the animals after injury and resuscitation, suggesting anesthetic recovery may have played a role in identifying the adverse events. The study had some potentially confounding features (see publication Moon-Massat et al. 2010), but limited resources prevented NMRC from further evaluating the causes of these adverse events.

*Sodium nitroprusside*. Elevations in systemic but not pulmonary arterial pressures were attenuated by co-infusion of sodium nitroprusside, at the dose tested (0.8 μg/kg/min), without significant group differences in other hemodynamics, tissue oxygenation, platelet function, coagulation, methemoglobin, or survival in a controlled hemorrhage swine model (Arnaud et al. 2012). The absence of a beneficial effect on pulmonary hypertension at the selected dose does not preclude a better effect at higher doses. The dose selected was within the normal clinical range, but a more comprehensive dose-response experiment may have found a dose able to attenuate HBOC-induced pulmonary vasoconstriction. Furthermore, this work was limited by the short 2 h-observation period without anesthetic recovery. This drug combination may warrant additional studies.

*Nitroglycerine*. An initial swine study of controlled hemorrhage was specifically designed to evaluate the cardiovascular and metabolic changes that occurred during resuscitation with HBOC-201 plus escalating doses of nitroglycerin (Katz et al. 2010; Manning 2009, Unpublished). The swine received equivalent volumes of fluid resuscitation as shed blood. Results demonstrated that the co-administration of 40 mcg/kg/min nitroglycerine during HBOC-201 resuscitation was the most effective in reducing HBOC-induced vasoconstriction. The combination reduced HBOC-201-induced increases in both systemic and pulmonary blood pressures as well as systemic and pulmonary vascular resistances, although, as with sodium nitrite, the effects were transient.

In a normovolemic, exercising swine model, co-infusion of nitroglycerine (titrated to maintain aortic blood pressure at pre-HBOC-201 baseline levels) virtually abolished the vasoconstrictor responses to HBOC-201 in the systemic and pulmonary vascular beds, while attenuating the coronary vasoconstrictor responses. This model, completely devoid of anesthetic or sedative drug effects, showed no clinical adverse effects and, in particular, no adverse cardiac effects despite exertion to 85 % of maximum oxygen consumption (Duncker 2009b, Unpublished). Translational survival studies were the next step to determine if this strategy of attenuating HBOC-201 vasoconstriction improved clinical outcome in hemorrhagic shock. The first uncontrolled hemorrhage study in swine also showed promising results. HBOC-201-induced vasoconstriction was attenuated with

5 μmol/kg/min nitroglycerine and there was a trend toward improved survival in a liver-injury uncontrolled hemorrhage model. The 72 h survival was 60 % (3/5) for HBOC-201 alone, 50 % (3/6) for HEX alone and 100 % (5/5) with HBOC-201 plus 5 μmol/kg/min nitroglycerine (McCarron 2012, Unpublished).

However, when FP-TBI was added to this model in the next study, the outcome changed (Scultetus et al. 2010). Although co-infusion of 2.5 μg/kg/min nitroglycerine (a reduced dose from the previous study) still resulted in attenuated HBOC-201-vasoconstriction, survival was compromised. Survival was 66 % (4/6) in HBOC-201, 16 % (1/6) with HBOC-201 + 2.5 μg/kg/min NTG, 0 % with HBOC + 5 μg/kg/min NTG and 0 % with HEX. This suggests that the TBI component in polytrauma affects the physiologic response to injury in different ways than hemorrhage alone. Consistent with this finding, the previously cited University of Washington study also found that co-infusion of NTG with HBOC-201 worsened survival time compared to HBOC-201 or LR in their high-pressure aortic tear plus TBI swine model (White et al. 2012).

These conflicting findings among injury models raise questions about resuscitation strategies in complex injuries. Any or all of several factors may contribute to these differences including the influence of wound type (low pressure vs. high pressure hemorrhage), vessel size, the vasoconstrictive properties of the resuscitation fluid, the volume of resuscitation fluid required, and the effect of TBI on normal compensatory responses.

## 27.6 Results of Other External Approaches to VA-HBOC-201

*Adenosine.* Adenosine, when titrated to maintain baseline blood pressures, safely and easily neutralized and attenuated, respectively, the vasoconstrictor responses of HBOC-201 in the systemic and pulmonary beds of awake, normovolemic swine even when exercising to 85 % maximal oxygen consumption. In addition, at these doses, adenosine produced vasodilation in excess of oxygen requirements in the coronary bed (Duncker 2009b, Unpublished).

*Drag reducing polymers.* Blood-soluble drag reducing polymers (DRPs), among them clinically available hyaluronic acid, offer an intriguing alternative mechanism to reduce the vasoconstricting effects of HBOCs. These drugs have been shown to produce effects on blood circulation, including an increase in tissue perfusion and tissue oxygenation and a decrease in vascular resistances (Bessa et al. 2011; Hu et al. 2011; Marhefka et al. 2009). Studies of RBC flow in artificial capillaries show that while red blood cells (RBCs) stream in the central lumen leaving a boundary layer of cell-free fluid adjacent to the capillary wall, the boundary layer narrows and the RBCs occupy a wider cross-section of the capillary when a DRP is added. By decreasing the volume of cell-free fluid in contact with vascular epithelium the addition of a DRP could hypothetically reduce the amount of HBOC in contact with the endothelial source of NO, thereby reducing NO scavenging and mitigating

vasoactivity. Because of its clinical availability, NMRC selected hyaluronic acid (HA) as a DRP to be tested as an HBOC-201 vasoactivity-mitigating additive but also evaluated polyethylene oxide due to the quantity of available literature on its use. The screening in vitro study at University of Pittsburgh demonstrated that hydrodynamic, rheological and microrheological performance of the two DRPs was not affected by the presence of HBOC-201 (Kameneva 2009).

Therefore, DRP additives could be successfully tested with HBOC products to determine if they reduce HBOC scavenging of NO and the resultant vasoconstriction selectively in the microcirculation. Theoretically, they would allow RBCs to relocate to the microvessel wall without affecting HBOC oxygen transport efficiency in capillaries.

## 27.7 Conclusions

The oxygen-carrying resuscitative fluid HBOC-201 has potential to benefit trauma casualties since it expands intravascular volume, stabilizes hemodynamics, transports and unloads oxygen, and increases tissue (including brain) oxygenation. It is universally compatible, is easy to administer, and does not require refrigeration; all characteristics that make it suitable for pre-hospital use. Collectively, the results of our HBOC-201 preclinical studies of solid organ hemorrhage demonstrated the largest benefit in survival with HBOC-201 occurs when the severity of the insult is high or the delay to definitive treatment is prolonged. In less extreme situations, standard resuscitation and compensatory physiological responses may be adequate for the injury. Despite its demonstrable efficacy in severe solid organ (low pressure) hemorrhage, it may be less beneficial in severe hemorrhage due to large artery (aorta) injury, either with or without concomitant TBI, and further study on the impact of the type of injury is necessary.

While the vasoconstrictive effect of HBOCs has been cited as a safety risk for this class of agents, it may be that this effect is of less concern and may even be beneficial, in the resuscitation of severe solid organ hemorrhage with or without TBI (see Table 27.1, Fig. 27.1). Nevertheless, various strategies to attenuate HBOC-201's vasoconstriction were attempted with some degree of success, particularly focusing on the use of concurrently infused nitroglycerin in swine hemorrhage models. Interestingly, when TBI was added to solid organ low pressure hemorrhage, attenuation of HBOC-201's vasoconstriction adversely impacted its ability to improve survival. More research will be necessary to understand the complex interplay among injury severity, hemorrhaging vessel size and type, HBOC-induced vasoconstriction, and the presence or absence of TBI.

The search continues for a safe, effective, and low logistic burden (volume, weight, storage requirements, etc.) trauma resuscitation fluid for pre-hospital use by military and civilian medical communities. The life-saving potential of such an agent is enormous. An FDA-approved HBOC for this indication may well be part of the solution for this crucial deficit in first-responder care.

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# Chapter 28
# Cellular-Type Hemoglobin-Based Oxygen Carrier as a Resuscitative Fluid for Hemorrhagic Shock: Acute and Long-Term Safety Evaluation Using Beagle Dogs

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## 28.1 Introduction

Blood transfusion is one of the most important measures in clinical medicine. However, some unresolved issues threaten the achievement of safe transfusion: the possibility of contamination of pathogens; mismatching of blood types; and numerous immunological difficulties. Guideline for safer blood transfusion has been revised repeatedly, such as the reduction of a transfusion trigger, the critical hemoglobin (Hb) level, to 6 g/dL to minimize unnecessary transfusion strictly or to avoid allogeneic transfusion as long as possible to prevent such side effects (American Society of Anesthesiologists Task Force 2006). In this respect, Hb-based oxygen carriers (HBOCs) are considered superior to allogeneic transfusion because they are free of blood-type antigens, and microbial pathogens. The stability of HBOCs for a long-term storage over years is also advantageous when

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compared with RBC transfusion (Sakai et al. 2008a). Considerably shorter half-life ($t_{1/2}$) of the HBOCs in the blood stream (2–3 days) limit their use (Lee et al. 1995), but they are applicable for shorter periods of use as: (1) a resuscitative fluid for hemorrhagic shock during an emergency situation temporarily or for bridging until RBCs are available (Johnson et al. 2001); (2) a fluid for preoperative hemodilution or perioperative $O_2$ supply fluid for a hemorrhage during elective surgery to avoid or delay allogeneic transfusion (Standl et al. 1998); (3) a priming solution for the circuit of an extracorporeal membrane oxygenator during cardiac surgery (Yamazaki et al. 2006); (4) an alternative use for other potential indications, for example, so-called $O_2$ therapeutics to oxygenate ischemic tissues (Contaldo et al. 2005; Nozue et al. 1996; Horinouchi et al. 2008).

A phospholipid vesicle or liposome-encapsulating concentrated human Hb (Hb-vesicle, HbV) is an HBOC (Djordjevich et al. 1987; Awasthi et al. 2004). HbV (particle diameter, approx. 250 nm) has characteristics that resemble those of natural RBCs because both have lipid bilayer membranes that prevent the direct contact of Hb with blood components and the endothelial lining, thus shielding all side effects of molecular Hb (D'Agnillo and Alayash 2001; Sakai et al. 2000a). Once in circulation, HbV particles are captured by the phagocytes in the reticuloendothelial system (RES) and are metabolized in the physiologically normal pathway after topload infusions (Sakai et al. 2001, 2004a, b; Sou et al. 2005). It was reported that the efficacy of HbV suspended in recombinant human serum albumin (rHSA) in extreme normovolemic hemodilution (80–90 % blood exchange) and resuscitation from hemorrhagic shock was proven (Izumi et al. 1997; Cabrales et al. 2005; Yoshizu et al. 2004; Sakai et al. 1997, 2004c). However, those experiments were mainly conducted with small animals. We couldn't evaluate the influence of HbV on pulmonary circulation in small animals. Therefore we conducted hemorrhage-resuscitation study using Beagle dog and reported the results (Yamamoto et al. 2012). In the previous report, observation was limited to four hours. Transient increase of pulmonary arterial pressure was observed in HbV group compare to the other groups that SAB(Shed Autologous Blood), rHSA(recombinant Human Serum Albumin), and LR (lactate Ringer solution) was used as resuscitative fluid. Splenectomy was performed in the previous study so that we could maintain uniform hemorrhagic shock state. In the present study, long term influence and safety as well as acute phase safety of HbV was studied. This time we preserved spleen to investigate the role of spleen in shock-resuscitation.

In the present study, we observed the animals for a long period (1 year) after resuscitation. Hemorrhagic shock was induced by 50 % bleeding (acute phase study) or 40 % bleeding (long term study). We analyzed systemic hemodynamics and $O_2$-transporting capacity within 4 h, and hematological, plasma biochemical, and histopathological examination within 1 year to clarify the impact on organ functions.

## 28.2 Materials and Methods

### *28.2.1 Preparation of HbVs Suspended in rHSA*

HbVs were prepared under sterile conditions, as reported in previous studies (Sou et al. 2003; Sakai et al. 2000b). The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dL) contained 14.7 mmol per L pyridoxal 5′-phosphate (PLP) (Sigma-Aldrich Co., St. Louis, MO) as an allosteric effector at a molar ratio of PLP/Hb of 2.5. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-$PEG_{5000}$ (NOF Corp., Tokyo, Japan), at a molar composition of 5/5/1/0.033. The lipopolysaccharide content, measured with a modified *Limulus* amebocyte lysate test, was less than 0.1 EU per mL (Sakai et al. 2004d). The physicochemical parameters are $P_{50}$, 27 Torr; 251 ± 81 nm particle diameter; and less than 3 percent MetHb content. Before use, the HbV suspension ([Hb] = 10 g/dL, 8.6 mL) was mixed with a solution of rHSA (25 g/dL, 1.4 mL; Nipro Corp. Osaka, Japan) to regulate the rHSA concentration in the suspending medium to 5 g per dL. Consequently, the Hb concentration became 8.6 g/dL. Under these conditions, the colloid osmotic pressure and the viscosity at 300 $s^{-1}$, 37 °C) of the HbV/rHSA were 20 mmHg and 2.9 cP, respectively.

### *28.2.2 Animal Preparation*

The Laboratory Animal Care and Use Committee of the School of Medicine, Keio University, approved the entire experimental protocol. The protocol complies with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council–National Academy of Sciences (Washington, DC: National Academy Press, 1996).

Experiments were carried out using 26 male beagle dogs (7.07 ± 0.33 kg b.w., NARC Corp., Chiba, Japan). The dogs were fasted 18 h before the experiment, but had free access to water up to 2 h before the anesthesia. The animals were bred in the cages individually. We used 10 beagle dogs for the acute phase study, and 16 beagle dogs for the chronic phase study. The dogs for the acute phase study were divided to HbV/rHSA group (n = 4), shed autologous blood (SAB) group (n = 3), and rHSA group (n = 3). The dogs for the chronic phase study were divided to HbV/rHSA group (n = 9) and SAB group (n = 7). The 9 dogs of the HbV/rHSA group were randomly divided into three and sacrificed at 28 days (n = 3), 168 days (n = 3), or 365 days (n = 3) after resuscitation experiment. Among 7 dogs of the SAB group, two dogs were randomly selected and sacrificed at 28 days, two dogs at 168 days, and the remained three dogs at 365 days after resuscitation.

### 28.2.3 Animal Preparation and Instrumentation

The animals, pre-medicated with atropine sulfate (0.07 mg/kg i.m.). Anesthesia was induced by intramuscular injection of ketamine hydrochloride (5 mg/kg i.m.). Animals were orally intubated, inhalation anesthesia was maintained with 2.0–2.5 %–sevoflurane mixed air supplied by an anesthesia apparatus (SN–487, Shinano Seisakusho Co., Tokyo, Japan). The concentration of sevoflurane (2.0–2.5 %) was adjusted as necessary to maintain the animal at a stable plane of anesthesia. Visual monitoring of spontaneous respiration was performed.

Electrocardiogram (EKG) electrodes were attached to the feet. A 5.5-F Thermodilution catheter (631Hf55; Edwards Lifescience, Irvine, CA, USA) was placed in the pulmonary artery via the right femoral vein for measurements of the mean pulmonary arterial pressure (MPAP), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), and cardiac output. The left femoral artery was cannulated to monitor arterial pressure as well as for blood sampling. The pressure line was connected to transducers (5100TW; Edwards Lifescience, Irvine, CA, USA), and these transducers and the EKG line were connected to a polygraph system (LEG-1000, Nihon Kohden Co., Tokyo, Japan). The right femoral artery was cannulated with a 16G I.V. cathter (Angiocath; Becton–Dickinson, Sandy, Utah, USA) to control the bleeding. $rSO_2$ (regional saturation of oxygen) was monitored using the $rSO_2$ monitor INVOS 4100 (Somanetics Inc., Troy, MI) at the forehead (brain $rSO_2$) and abdomen (rectus abdominis muscle $rSO_2$).

### 28.2.4 Experimental Protocol

After establishment of stable anesthesia, animals were randomly assigned to three experimental groups in acute study, i.e. shed autologous blood (SAB) group ($n = 3$), 5 g/L recombinant human serum albumin in Saline (rHSA) group ($n = 3$), and HbV suspended in 5 % rHSA/saline solution (HbV/rHSA) group ($n = 4$). In chronic study, dogs were assigned to two groups, i.e. shed autologous blood (SAB) group ($n = 7$), and HbV suspended in 5 % rHSA/saline solution (HbV/rHSA) group ($n = 9$).

The systemic blood volume was estimated to be 86 mL per kg of the total body weight. In acute phase study, a 50 % volume of the circulation blood was withdrawn from the right femoral artery cathter at a rate of 20 ml/min. In the chronic phase study, a 40 % volume was withdrawn. Withdrawn blood was preserved in a several 50 ml syringe containing 7 ml of CPD solution (Karmi C, Kawasumi Laborataories Inc. Tokyo, Japan) in SAB group.

The hemorrhagic shock state was maintained for 1 h. Thereafter, designated isovolemic resuscitative fluid was injected intravenously. In all experiment, infusion rate of resuscitative fluid were maintained at 20 mL/kg/min. After resuscitation, no additional intravenous fluid was allowed except for the cold 5 % glucose required to measure cardiac output.

### 28.2.5 Measurements

In the acute phase study, 0.5 mL of arterial blood and 2.0 mL of mixed–venous blood were collected from the femoral and pulmonary arteries at the following ten time–points: before hemorrhage, immediately after hemorrhage, 1 h after the shock, immediately after resuscitation, and 0.5, 1, 1.5, 2, 3 and 4 h after resuscitation. In the chronic phase study, 10 mL of venous blood was collected from cepharic vein at the following ten time–points: before the bleeding, and 1, 3, 7, 14, 28, 56, 84, 168 and 365 days after resuscitation.

MAP was monitored through the right femoral artery, and MPAP, PCWP and CVP were through the flow directed pulmonary artery catheter connected to a transducer (5100TW, Edwards Lifesciences, Irvine, CA., USA). These transducer and EKG line were connected to a polygraph system (PEG-1000, Nihon Kohden, Tokyo, Japan). MAP, MPAP and HR were continuously monitored, and cardiac output was assessed by a thermodilution procedure with the rapid injection of cold saline (5 mL, 4 °C) in duplicate using a cardiac output measurement apparatus (Vigilance system, Edwards Critical-Care Division Irvine, CA, USA). The systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) were calculated as $SVR = 79.92 \times (MAP - CVP)/\text{cardiac output}$, and $PVR = 79.92 \times (MPAP - PCWP)/\text{cardiac output}$, respectively. MAP, MPAP, PCWP, CVP, cardiac output and $PtO_2(R)$ were measured at the same points stated above.

Withdrawn blood specimen (approximately 1 mL) was rapidly applied to a blood gas system (ABL555, Radio Meter Trading, Copenhagen, Denmark) to measure the pH, $O_2$ pressure ($PaO_2$) and $CO_2$ pressure ($PaCO_2$) of the arterial blood, and the $O_2$ pressure ($PvO_2$) and lactic acid level of the venous blood. Arterial $O_2$-saturation ($SaO_2$) and mixed venous $O_2$-saturation ($SvO_2$) were calculated by $PaO_2$ and $PvO_2$, respectively, using an $O_2$-equilibrium curve of the canine RBC, which was measured by a Hemox Analyzer (TCS medical products, Philadelphia, USA).

The hematocrit (Hct) was measured by using the glass capillary and centrifugation (4,500 rpm, 5 min). The Hb concentration of the arterial blood was obtained using a multi-system automatic blood cell counter (KX-21, Sysmex, Kobe, Japan). The presence of HbV in the blood interferes in the measurement of Hb concentration, thereafter the Hb concentration of the HbV/rHSA group was measured with a modified cyanomet-hemoglobin method.

The plasma Hb concentration derived from HbV was calculated as follows. Blood samples from the venous line after the HbV infusion were centrifuged at 4 °C (3,500 rpm, 10 min). The HbV molecule in the supernatant was converted to the cyanomet form using a Hemoglobin Test Wako (Wako Pure Chemical Industries, Ltd., Tokyo), and its concentration was determined by the absorption spectral measurement using a UV–vis absorption spectrophotometer (V-570, JASCO, Tokyo, Japan). The percentage of metHb within the vesicles was periodically calculated by the ratio of absorbance at 405 nm (metHb) and 430 nm

(deoxyHb) in the Soret band using a UV–vis spectrometer without destruction of the HbV (Atoji et al. 2006).

The arterial $O_2$-content ($CaO_2$ (RBC)) of the beagle dog's red blood cell (RBC) was estimated by the following equation. The oxygen delivery ($DO_2$ (RBC)) of the beagle dog's RBC was calculated as the product of Qt and $CaO_2$ (RBC).

$$CaO_2(\mathrm{RBC}) = [\mathrm{Hb}]_{\mathrm{RBC}} \times 1.34 \times \left\{SaO_2 \times 10^{-2}\right\}$$

$$DO_2(\mathrm{RBC}) = CaO_2(\mathrm{RBC}) \times 10 \times \mathrm{Qt}$$

The arterial $O_2$-content ($CaO_2$ (HbV)) of HbV was estimated by the following equation. The oxygen delivery ($DO_2$ (HbV)) of HbV was calculated as the product of the cardiac output (Qt) and $CaO_2$ (HbV).

$$\begin{aligned} CaO_2(\mathrm{HbV}) &= [\mathrm{Hb}] \times 1.34 \times \left\{1 - \text{metHb percentage} \times 10^{-2}\right\} \\ &\quad \times \left\{SaO_2(\mathrm{HbV}) \times 10^{-2}\right\} \\ DO_2(\mathrm{HbV}) &= CaO_2(\mathrm{HbV}) \times 10 \times \mathrm{Qt} \end{aligned}$$

The arterial $O_2$-content ($CaO_2$ (DO)) of the dissolved oxygen was estimated by the following equation. The oxygen delivery ($DO_2$ (DO)) of the dissolved oxygen was calculated as the product of Qt and $CaO_2$ (DO).

$$CaO_2(\mathrm{DO}) = 0.003 \times PaO_2$$

$$DO_2(\mathrm{DO}) = CaO_2(\mathrm{DO}) \times 10 \times \mathrm{Qt}$$

The total oxygen delivery ($DO_2$) was calculated by the following equation.

$$DO_2 = DO_2(\mathrm{RBC}) + DO_2(\mathrm{HbV}) + DO_2(\mathrm{DO})$$

The mixed venous $O_2$-content ($CvO_2$ (RBC)) of the beagle dog's red blood cell (RBC) was estimated by the following equation. The oxygen consumption ($VO_2$ (RBC)) of the beagle dog's RBC was calculated as the product of Qt and the difference between $CaO_2$ (RBC) and $CvO_2$ (RBC).

$$\begin{aligned} CvO_2(\mathrm{RBC}) &= [\mathrm{Hb}]_{\mathrm{RBC}} \times 1.34 \times \left\{SvO_2(\mathrm{RBC}) \times 10^{-2}\right\} \\ VO_2(\mathrm{RBC}) &= \left\{CaO_2(\mathrm{RBC}) - CvO_2(\mathrm{RBC})\right\} \times 10 \times \mathrm{Qt} \end{aligned}$$

The mixed venous $O_2$-content ($CvO_2$ (HbV)) of HbV was estimated by the following equation. The oxygen consumption ($VO_2$ (HbV)) of HbV was calculated as the product of Qt and the difference between $CaO_2$ (HbV) and $CvO_2$ (HbV).

$$\begin{aligned} CvO_2(\mathrm{HbV}) &= [\mathrm{Hb}] \times 1.34 \times \left\{1 - \text{metHb percentage} \times 10^{-2}\right\} \\ &\quad \times \left\{SvO_2(\mathrm{HbV}) \times 10^{-2}\right\} \\ VO_2(\mathrm{HbV}) &= \left\{CaO_2(\mathrm{HbV}) - CvO_2(\mathrm{HbV})\right\} \times 10 \times \mathrm{Qt} \end{aligned}$$

The mixed venous $O_2$-content ($CVO_2$ (DO)) of the dissolved oxygen was estimated by the following equation. The oxygen consumption ($VO_2$ (DO)) of the dissolved oxygen was calculated as the product of Qt and the difference between $Cao_2$ (DO) and $CVO_2$ (DO).

$$\mathrm{CvO_2(DO)} = 0.003 \times \mathrm{PvO_2}$$
$$\mathrm{VO_2(DO)} = \{\mathrm{CaO_2(DO)} - \mathrm{CvO_2(DO)}\} \times 10 \times \mathrm{Qt}$$

The total oxygen consumption ($VO_2$) was calculated by the following equation.

$$\mathrm{VO_2} = \mathrm{VO_2(RBC)} + \mathrm{VO_2(HbV)} + \mathrm{VO_2(DO)}$$

In the chronic phase study, the collected venous blood was used for blood cell counts with an automatic blood cell counter. The rest of the blood was centrifuged (5,000 rpm, 10 min) to separate the plasma which was then ultracentrifuged (50,000 rpm, 20 min) to sediment the HbV particles from the plasma at 1, 3 and 7 days after the resuscitation with HbV/rHSA to avoid their interference by HbV particles in the plasma biochemical assays (Sakai et al. 2003). The obtained transparent serum specimens were stored at –80 °C until biochemical tests (Biken, Kyoto, Japan). The selected analyses were aspartate aminotransferase phosphatase (ALP), $\gamma$-glutamylteansferase ($\gamma$-GTP), cholinesterase (ChE), total protein (TP), albumin (ALB), creatine phosphokinase (CPK), amylase (AMY), lipase, leucine aminopeptidase (LAP), urea nitrogen (BUN), creatinine (Cre), uric acid (UA), total cholesterol (T-chol), free cholesterol (F-chol), high density lipoprotein cholesterol (HDL-chol), triglyceride (TG), free fatty acid (FFA), phospholipids, total lipids, total bilirubin (T-Bil), Fe, Cu, K, Ca, inorganic phosphate (IP), and Mg.

### *28.2.6 Histopahological Examination*

The animals were finally euthanized with large dose of pentobarbital and exsanguination. Then autopsy was performed to get the specimen of organs (esophagus, small intestine, large intestine, liver, pancreas, spleen, thymus, lung, trachea, heart, kidney, testis, and adrenal) were obtained for a histopahological study. They were fixed in a 10 % formalin neutral buffer solution (Wako Pure Chemicals, Osaka, Japan) immediately after removal, and the paraffin sections were stained with hematoxylin & eosin (Mitsubishi Chemical Safety Institute, Kumamoto, Japan).

### *28.2.7 Statistical Analyses*

Data are reported as mean ± standard deviation (SD) for all measurements. Data were analyzed using Stat View (SAS Institute, Inc., Cary, N.C., USA). Differences compared with the control (baseline) group were analyzed with paired *t* test, and

differences between the groups were analyzed with Mann–Whitney U test. The changes were considered significant if the $p$ value was less than 0.05 in the acute phase study, and 0.01 in the chronic phase study.

## 28.3 Results

### *28.3.1 Acute Phase Study*

Beagle dogs of all groups tolerated well the 50 % bleeding inducing hemorrhagic shock and resuscitation. They survived for 4 h after the resuscitation without any change in their appearance.

Circulation

MAP before hemorrhage was 102 ± 19 mmHg on the average; it decreased significantly to 19 ± 5 mmHg immediately after hemorrhage (Fig. 28.1). After resuscitation, both the SAB and HbV/rHSA groups showed immediate recovery and stable values for the 4 h. The rHSA group showed significantly lower MAP than the HbV/rHSA group at 1, 3, and 4 h after infusion.

All groups showed significantly higher MPAP immediately after infusion than the baseline values. However, there were not significant differences between groups. After that, all groups showed almost stable values. The HbV/rHSA group showed slightly lower PCWP after resuscitation than the other groups. The SAB group showed slightly higher CVP after infusion than the other groups.

There was no significant change in the time course of the HR during the experiment. CO before hemorrhage was 1.4 ± 0.3 L/min on the average; it decreased significantly to 0.2 ± 0.1 L/min immediately after hemorrhage. After resuscitation, all groups showed immediate recovery, and rHSA group showed significantly higher values than the baseline. The SAB group showed significantly lower values than the HbV/rHSA group at 0.5, 1, 1.5, and 3 h after resuscitation.

All groups showed significantly lower SVR at 0, 0.5, 1, 1.5, and 2 h after resuscitation than the baseline values. The rHSA group showed lower SVR than the HbV/rHSA group at 0, 0.5, 1, 1.5, 2 h after infusion. The 50 % hemorrhage increased the PVR, however, after the hemorrhage, all groups showed stable values for 4 h of the observation period.

Blood gas analysis (Fig. 28.2).

The pH value decreased to 7.13–7.22 after hemorrhage, but both the SAB and HbV/rHSA groups showed immediate recovery and stable values for the 4 h. The rHSA group tended to recover late. BE decreased to 11.5–15.6 mmol/L after hemorrhage, but all groups showed gradual recovery to the initial level after infusion. The lactic acid decreased to 3.07–5.07 mmol/L after hemorrhage, but all groups showed gradual recovery like BE. As a result of the hyperventilation, the slight elevation of $PaO_2$ and the decline of $PaCO_2$ were seen after hemorrhage. However, all groups showed recovery and similar tendency. $PvO_2$ before

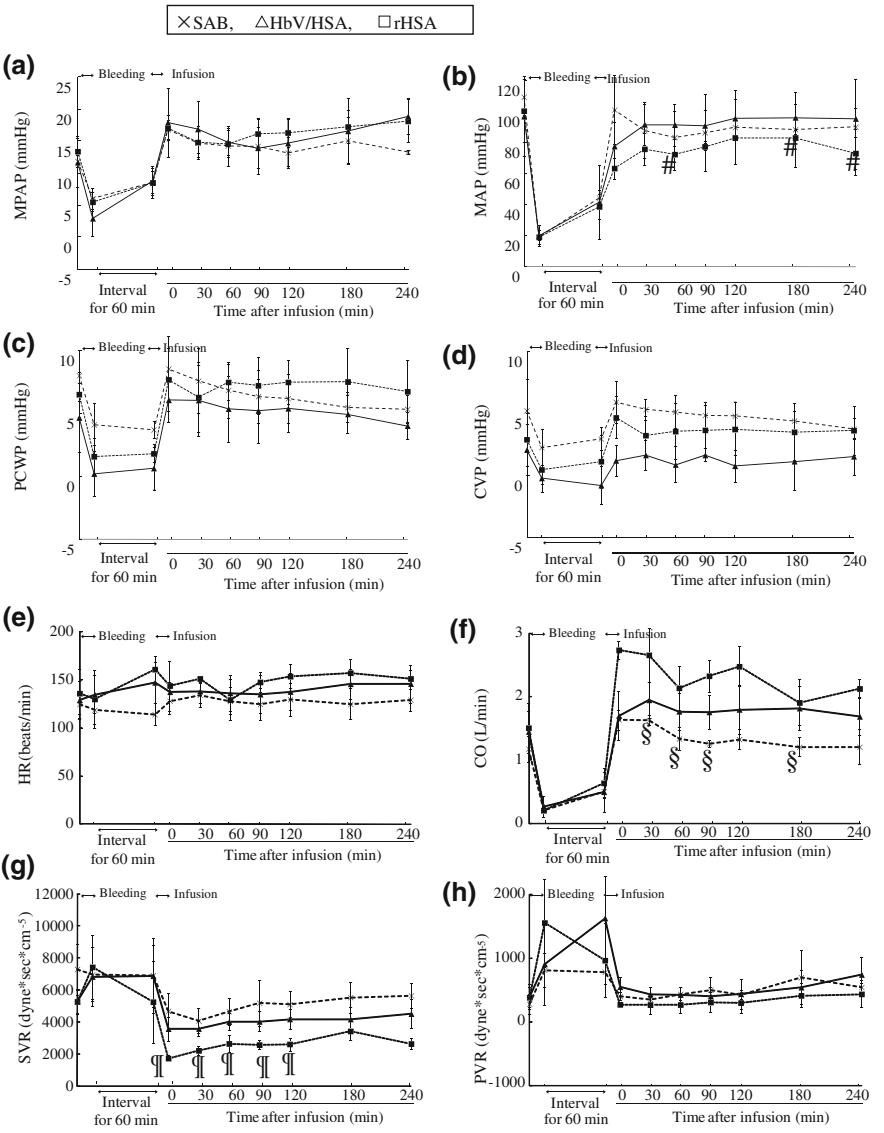


**Fig. 28.1** Changes in mean pulmonary arterial pressure (MPAP), mean arterial pressure (MAP), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), heart rate (HR), cardiac output (CO), systemic vascular resistance (SVR), and pulmonary vascular resistance (PVR) during hemorrhagic shock and resuscitation with infusion of rHSA alone, shed SAB and HbV/rHSA. The values are mean ± SD. #,§: significantly different between HbV/rHSA group ($p < 0.05$)

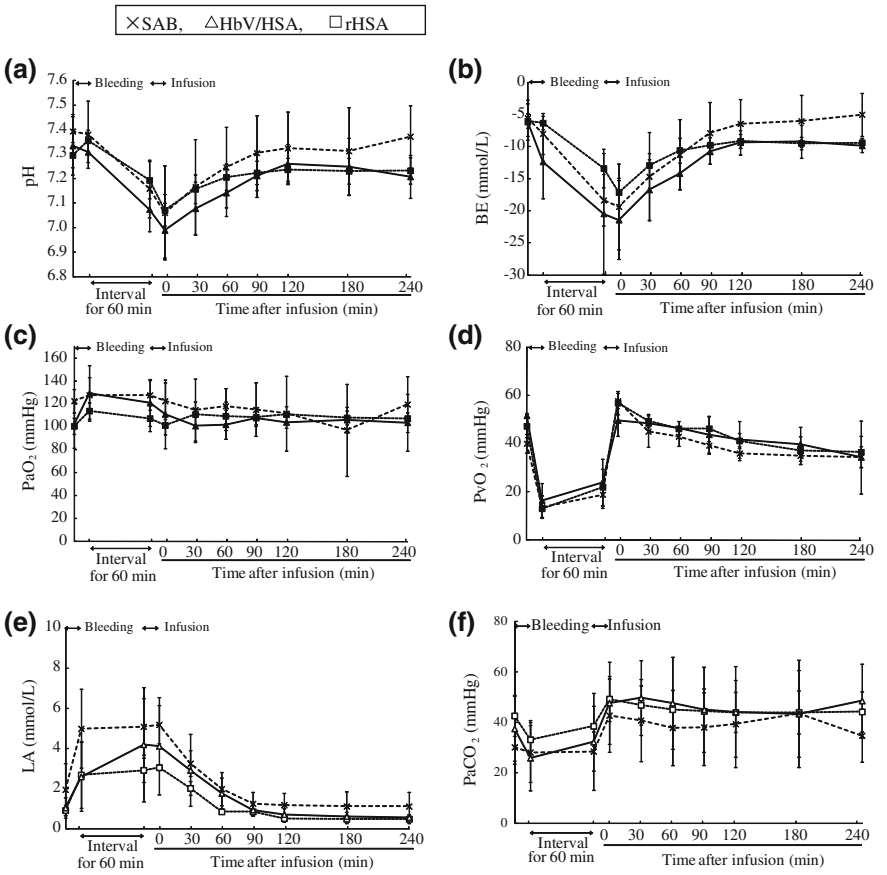


**Fig. 28.2** Changes in pH, $PaO_2$, $PaCO_2$, base excess (BE), lactate, and $PvO_2$ during hemorrhagic shock and resuscitation with infusion of rHSA alone, SAB and HbV/rHSA. The values are mean ± SD. There was no significant difference between rHSA or SAB and HbV/rHSA group

hemorrhage was 48 ± 5 Torr on the average; it decreased significantly to 15 ± 6 Torr immediately after hemorrhage. After resuscitation, all groups showed immediate recovery and similar tendency.

Hematology (Fig. 28.3).

There was no significant change after bleeding in Hct for all groups. As a result of the dilution of blood, the rHSA and HbV/rHSA groups showed significantly lower values than the baseline values, while the SAB group showed higher values during the experiment (Fig. 28.3c). In the HbV/rHSA group the total Hb levels before hemorrhage was 10.4 ± 1.6 g/dL, and after resuscitation it was 13.8 ± 1.6 g/dL at 0 h, and 9.9 ± 1.2 g/dL at 4 h (Fig. 28.3b). The concentration of Hb derived from HbV was 4.2 ± 0.5 g/dL (37.5 ± 4.5 % of total Hb) at 0 h,

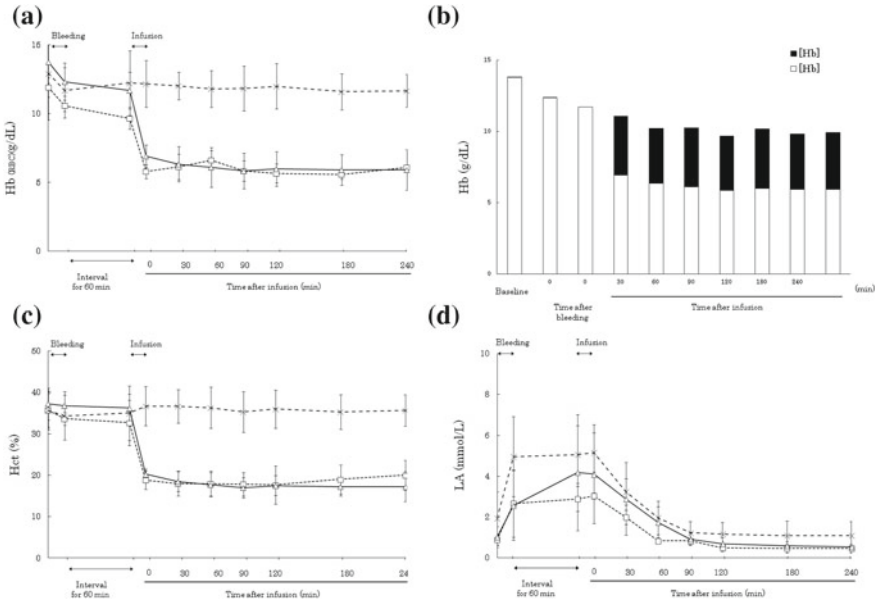


**Fig. 28.3** Change in hemoglobin (Hb) concentration, hematocrit (Hct), and lactic acid (LA) level during hemorrhagic shock and resuscitation with infusion of rHSA alone, SAB, and HbV/rHSA. The values are mean ± SD. Composition of Hb concentration in the whole blood in HbV/rHSA group (B)

and 4.0 ± 0.8 g/dL (40.5 ± 8.1 % of total Hb) at 4 h. The level of metHbV increased to 9.1 ± 3.0 % at 4 h.

Oxygen delivery and consumption (Fig. 28.4).

As regards to the oxygen delivery and consumption, the rHSA group tended to show lower $DO_2$ than the other groups after the resuscitation, and showed significantly lower value than HbV/rHSA group 3 h after resuscitation (Fig. 28.4a). In the HbV/rHSA group, $DO_2$(HbV) was 34–38 % of the total $DO_2$. Oxygen consumption was not significantly different between HbV/rHSA group and SAB, or rHSA group. In HbV/rHSA group $VO_2$(HbV) showed 26–29 % of the total $VO_2$.

### *28.3.2 Long Term Study*

Beagle dogs of all groups tolerated well the 40 % bleeding inducing hemorrhagic shock and resuscitation. They survived without any change in their appearance until their intentional sacrifice. The body weight of beagle dogs before resuscitation was 7.2 ± 0.3 kg, which increased monotonously to 12 ± 1.8 kg at one year after resuscitation in both groups (Fig. 28.5). The Hct before the resuscitation

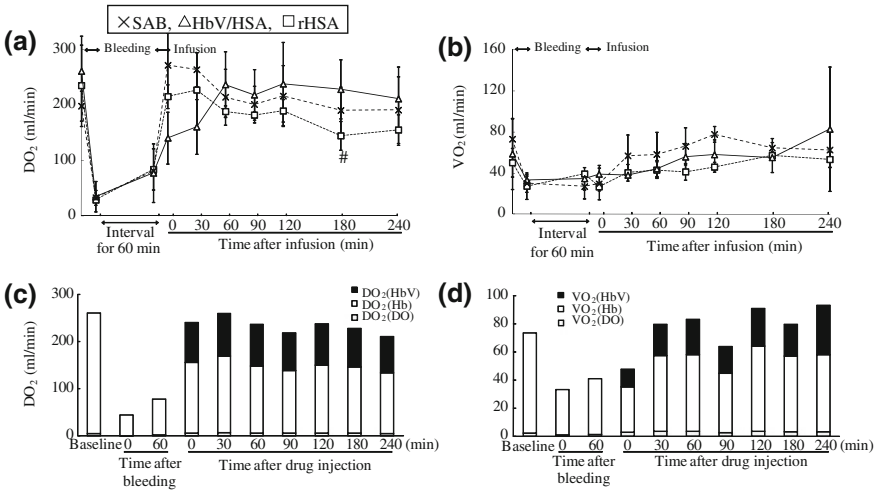


**Fig. 28.4** Changes in oxygen delivery ($DO_2$), and oxygen consumption ($VO_2$) during hemorrhagic shock and resuscitation with infusion of rHSA alone, sSAB and HbV/rHSA (*Top*). The rates of oxygen delivery derived from dissolved oxygen in plasma ($DO_2$(Plasma)), hemoglobin of RBCs ($DO_2$(RBC)), and HbV ($DO_2$(HbV)) in total oxygen delivery ($DO_2$) of the HbV/rHSA group and oxygen consumption derived from dissolved oxygen in plasma ($VO_2$(Plasma)), hemoglobin of RBCs ($VO_2$(RBC)), and HbV ($VO_2$(HbV)) in total oxygen consumption ($VO_2$) of the HbV/rHSA group (*Bottom*). The values are mean ± SD. # significantly different versus the HbV/rHSA group ($p < 0.05$)

was approximately 35 %. It decreased to about 29 % for the HbV/rHSA group after recovery from acute phase study. However, it showed monotonic increase; at 7 days, the Hct showed a complete recovery to the baseline level (about 35 %). Although Ht recovered consistently in HbV/rHSA group, we could see distinguished significances until 2 months. White blood cell and platelet counts showed non-significant changes between the HbV/rHSA and SAB groups, and then maintained rather steady values.

Regarding the plasma biochemical tests, AST and ALT showed increases on Day 1 in both HbV/rHSA and SAB groups, but it reverted to the original level on Day 3 (Fig. 28.5). LDH showed decreases on Day 1 in both groups, but both groups showed gradual increases until Day 7. The HbV/rHSA group tended to show lower ALP than the SAB group, and showed significantly lower values than baseline after 168 and 365 days. γ-GTP, ChE, TP, and ALB showed stable values for 1 year. CPK showed increases on Day1 in both groups, but it reverted to the original level on Day 3. Amylase showed non-significant change between HbV/rHSA and SAB group for 1 year. Lipase showed significant decrease in HbV/rHSA group on Day 7, but it tended to show gradual recovery. LAP, BUN, Cre, and UA showed non-significant changes between HbV/rHSA and SAB group. Regarding plasma lipid components in the HbV/rHSA group, Total-cholesterol level and Free-cholesterol level showed significant increases on Day 7. However,

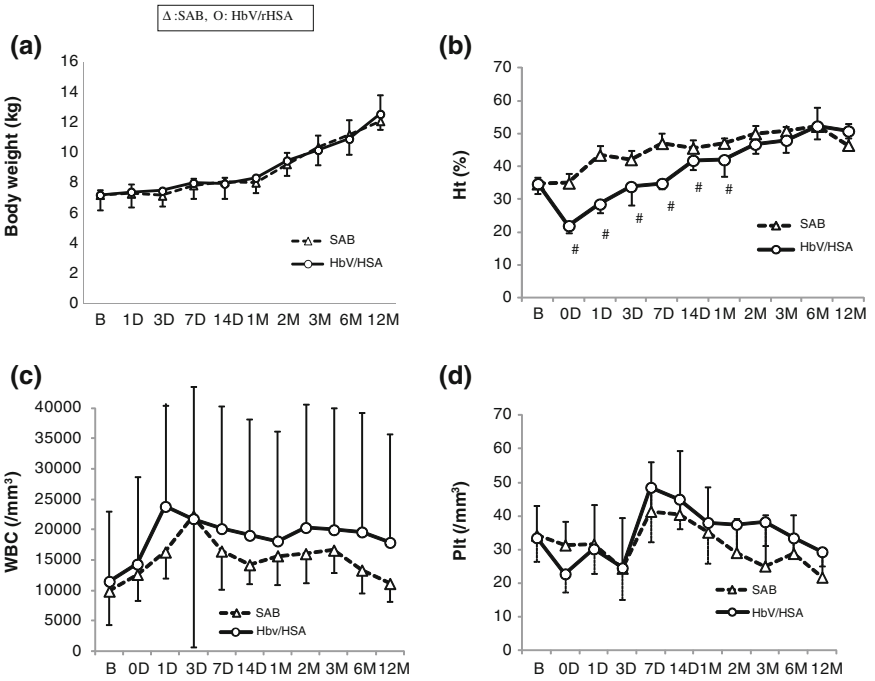


**Fig. 28.5** One-year observation of changes in body weight, hematocrit (Hct), white blood cells count (WBC), and platelet count (PLT) after resuscitation with infusion of SAB and HbV/rHSA. The values are mean ± SD. # significantly different versus the autologous shed blood group ($p < 0.01$)

they returned to their original levels at Day 14 (Fig. 28.6). HDL-Chol (High Density Lipoprotein-cholesterol) showed significant decrease for HbV/rHSA group on Day 1, but it reverted to the original level on Day 3. Trigriseride (TG) and Free Fatty Acid (FFA) showed non-significant change between the HbV/rHSA and SAB groups. Phospholipids and Total Lipid showed significant increases in the HbV/rHSA group on Day 7, but they showed non-significant change after Day 3. Total bilirubin (T-Bil) and Fe maintained steady values. Copper ion showed significant increase in the SAB group on Day 1, but it showed similar tendency after Day 3. K, Ca, IP, and Mg showed stable values for 1 year.

### *28.3.3 Histopathological Study*

In acute phase study, sinusoid of the liver showed the eosinophilic fine granular material in the HbV/rHSA group (Fig. 28.7). The red pulp zone of the spleen showed eosinophilic fine granular material in the HbV/rHSA group. These findings

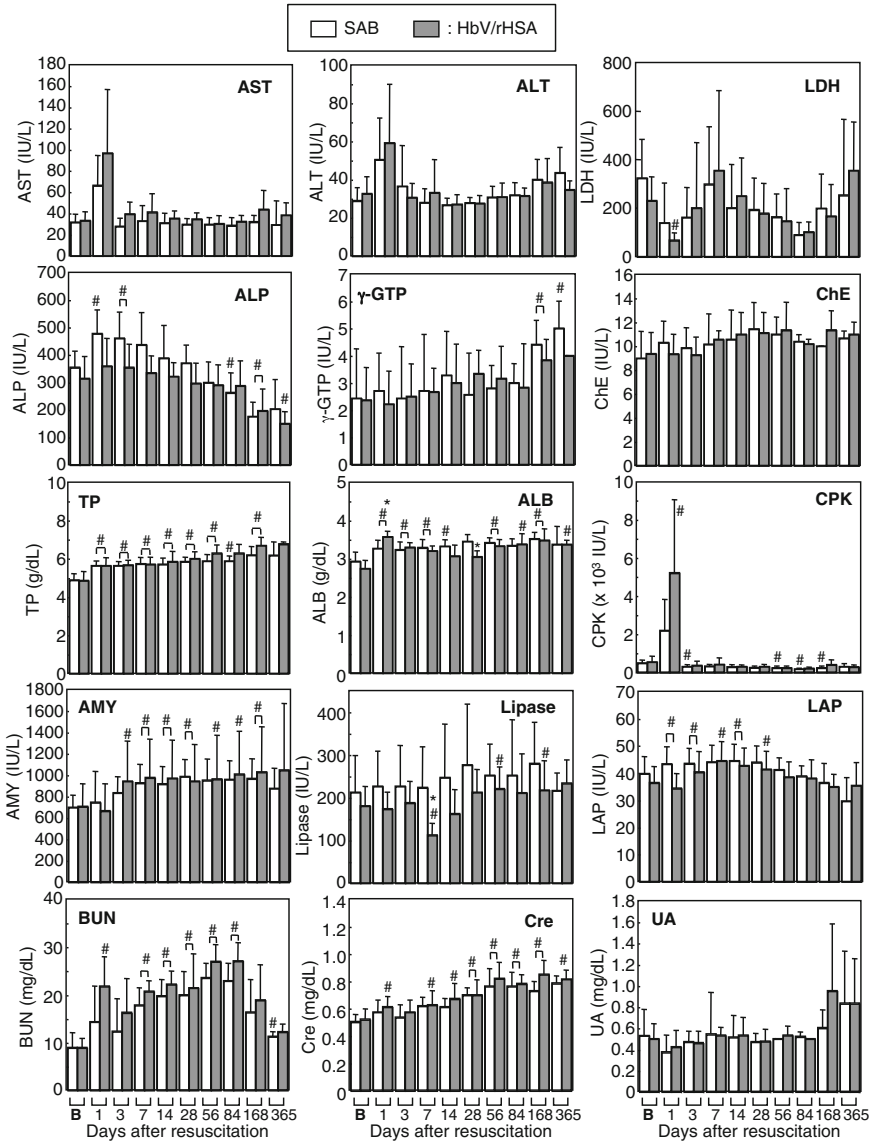


**Fig. 28.6** Plasma biochemical tests reflecting the organ functions such as liver, pancreas, and kidneys during one year after 40 percent hemorrhagic shock and resuscitation with infusion of SAB and HbV/rHSA. The values are mean ± SD. # Significantly different from baseline ($p < 0.01$); * significantly different versus the autologous shed blood group ($p < 0.01$). *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactate dehydrogenase, *ALP* alkaline phosphatase, *GGTP* γ-glutamyltransferase, *ChE* cholinesterase, *TP* total protein, *ALB* albumin, *CPK* creatine phosphokinase, *AMY* amylase, *LAP* leucin amino peptidase, *BUN* blood urea nitrogen, *Cre* creatinine, *UA* uric acid

were considered significant amounts of HbV phagocytized by macrophages in the spleen and Kupffer cells in the liver were observed. In chronic phase study, the HbV/rHSA group on Day 28 showed brown pigment deposition in the spleen and the Kuppfer cells of the liver (Fig. 28.8). These findings were not observed at other time points of the HbV/rHSA groups and SAB groups. No significant changes were seen in the pancreas, lung, heart, and kidney (Fig. 28.9).

## 28.4 Discussion

Our primary finding in this study is that HbV suspended in an albumin solution showed a similar resuscitative ability to that of SAB. Cardiovascular function such as MAP, PAP, CVP, PCWP recovered after resuscitation, and there was not significant differences between all groups (HbV/rHSA group, rHSA group, and SAB group). We have reported the efficacy as a resuscitative fluid in hemorrhagic shock in canine model (Yamamoto et al. 2012). In the previous report, up-regulation of PAP in HbV group after resuscitation was significantly higher than the other groups (SAB, rHSA, and Lactate Ringer solution groups). We thought HbV has the constrictive potential to the pulmonary circulation. In this study, PAP recovered after resuscitation in all groups and there were no significant differences between groups. From these findings, we considered that spleen plays a primary role to mitigate the influence of HbV. Since spleen is a large RES organ, it could play a role as a filter of the particle that might influence on endothelium. Furthermore, it was the first time to clarify the long term safety of HbV using canine model as evidenced by the result that HbV did not disturb the cardiopulmonary circulation, and all the dogs survived for 1 year without any remarkable side effect for each organs.

In the acute phase study, the rHSA group tended to delay the recovery of decreased MAP, however, HbV/rHSA group showed the prompt response that was similar to SAB group. The change of $DO_2$ showed the similar characteristics to the change of MAP, and HbV contributed 26–29 % of the total $DO_2$ values. These findings showed that the resuscitative ability of HbV was better than that of rHSA and was equivalent to SAB, because of the enough ability of oxygen carrying capacity. Regarding the rHSA group, MAP tended to show lower values than the other groups, and SVR showed significantly lower value than the HbV/rHSA group. The remarkable increase of cardiac output (CO) was caused by the reduced blood viscosity and the increased HR to compensate for the inadequate oxygen carrying capacity. Due to the sufficient compensatory mechanism to this level of hemorrhagic shock and resuscitation, the blood gas parameters showed non-significant changes between rHSA and the other groups. By contrast, lactic acid showed lower value in the rHSA group than in other groups. Because all the dogs in the rHSA groups survived for 4 h, the rHSA fluid alone possesses a resuscitative ability to restore blood volume in spite of the lack of oxygen carrying capacity. Unlike a human spleen, a canine spleen is an important blood reservoir capable of

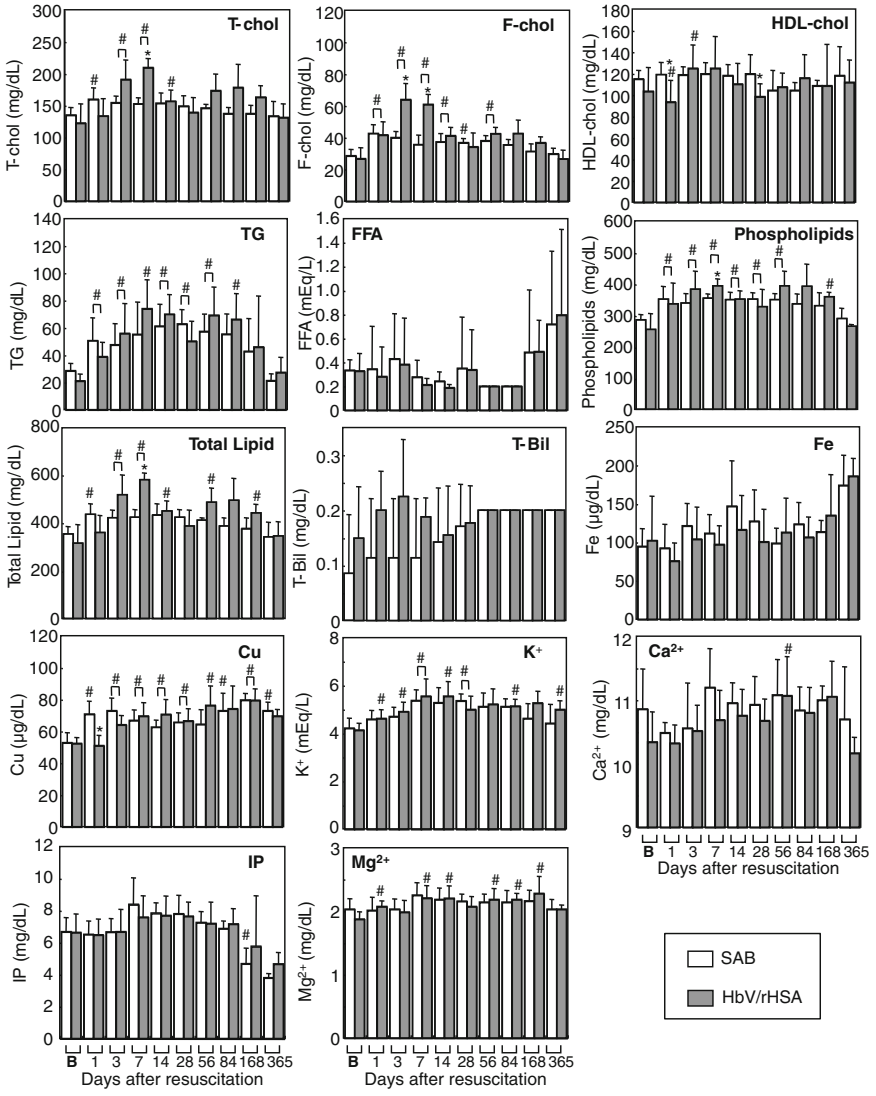


**Fig. 28.7** Plasma biochemical tests relating the metabolism of the components of HbV (lipids and Hb), microelements, and electrolytes during one year after 40 percent hemorrhagic shock and resuscitation with infusion of SAB and HbV/rHSA. The values are mean ± SD. # Significantly different from baseline ($p < 0.01$); * significantly different versus the autologous shed blood group ($p < 0.01$). *T-chol* total cholesterol, *F-chol* free cholesterol, *HDL-chol* high density lipoprotein cholesterol, *TG* triglyceride, *FFA* free fatty acid, *T-Bil* total bilirubin, *IP* inorganic phosphate

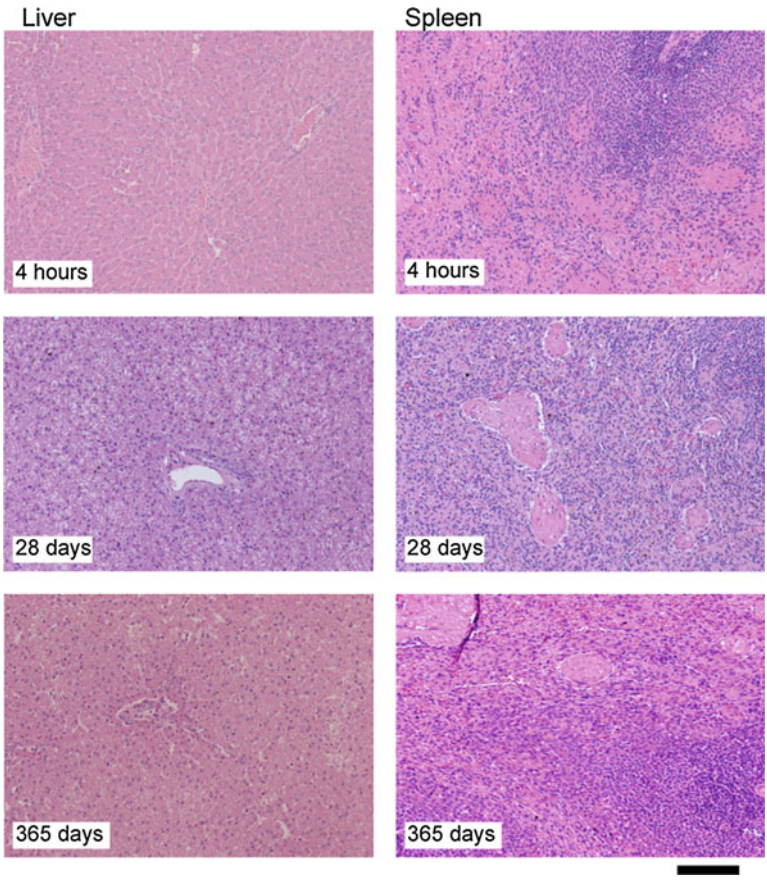


**Fig. 28.8** Histology of spleen and liver of HbV/rHSA group at 4 h, and 28 and 365 days after resuscitation. The presence of spleen macrophages and liver Kupffer cells phagocytizing HbV particles was shown at 4 h. The liver and spleen at 28 days contained slight brown pigment deposition. No significant change is noted at 365 days. Scale bar, 100 μm. Hematoxylin and eosin stains

maintaining Hct at a stable level by "autotransfusion" in response to a blood loss such as hemorrhagic shock (Hoit et al. 1991). This might have caused the unexpectedly moderate resuscitative ability of the rHSA solution and without causing severe shock state. In previous report, while animals undertook splenectomy and 50 % hemorrhagic shock, in rHSA group, animals could maintain proper lactate level and showed good recovery of pH during resuscitation and observation phase (Yamamoto et al. 2012). In this respect, resuscitation potential of albumin solution was high, given the patient had enough cardiac reserve to restore organ perfusion, preventing hypoxia. In HbV/rHSA group, cardiac output, heart rate, and lactate level showed almost identical change compared with SAB group.

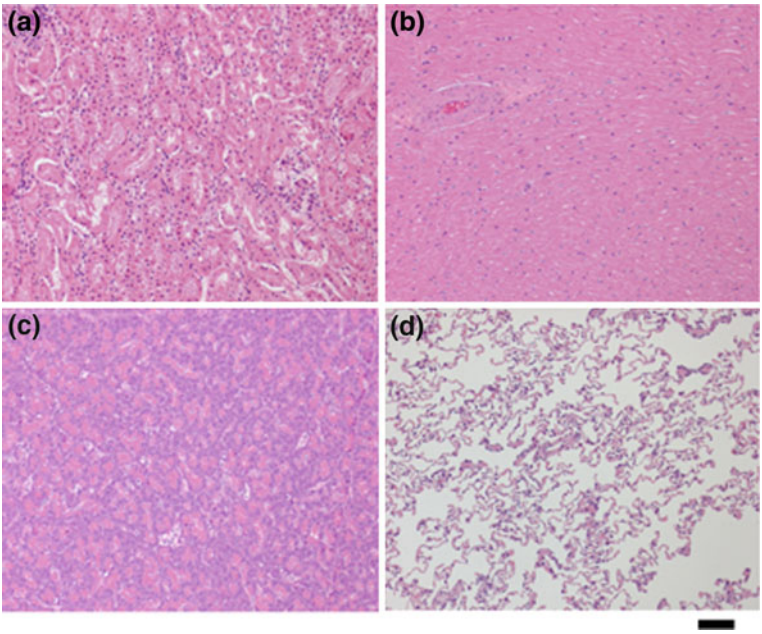


**Fig. 28.9** Histology of kidneys (**a**), heart (**b**), pancreas (**c**), and lungs (**d**) of HbV/rHSA group at 365 days after resuscitation. No significant change is noted in these organs. Scale bar, 100 μm. Hematoxylin and eosin stains

It has to be emphasized that acute and chronic safety of HbV was shown in this study using the canine model in which a large amount of HbV was transfused.

It has been reported that resuscitation from hemorrhagic shock with acellular type HBOCs such as polymerized or intramolecular cross-linked Hb causes the elevation of MAP beyond the baseline values. The hypertension may be presumably due to the high affinity for nitric oxide of acellular type HBOCs and their smaller size that enables nitric oxide trapping in the proximity of the endothelium (Sakai et al. 2000a; Yu et al. 2008; Nakai et al. 1998; Driessen et al. 2001; Kasper et al. 1998; Natanson et al. 2008). In this study, the abnormal increase of MAP after infusion of resuscitative fluid was not seen. MPAP showed increase immediately after the infusion, and gradual decrease after 30 min from resuscitation. PVR showed the stable values after the infusion. As HbV/rHSA group did not show the specific elevation of MAP, MPAP, SVR and PVR, we considered that the cellular HbV presumably trap nitric oxide slowly as erythrocyte does. (Sakai et al. 2011; Arazov et al. 2011). On the other hand, it has been reported that the infusion of the other cellular type HBOCs cause the elevation of PVR in a beagle dog model and the elevation of SVR in a goat model (Pape et al. 2008; Kansaku et al. 2008). These circulatory abnormalities are not related the high affinity for nitric oxide because Hb is encapsulated, but may be presumably due to the activation of complement and platelet caused by the lipid component of the membrane

encapsulating the Hb (Abe et al. 2006; Sou and Tsuchida 2008). In the previous report, 50 % hemorrhagic shock model in beagle dog that underwent splenectomy showed transient increase of PAP after resuscitation while in the present study we could not find this change. The difference between previous study and acute phase of the present study is whether splenectomy was conducted. Spleen might have the ability to compromise the transient increase of PAP during resuscitation. Further study is required.

Pathological examination of the liver and spleen of 4 h after resuscitation showed accumulation of HbV (Sakai et al. 2001). Because the circulation half-life of HbV is about 35 h (Sou et al. 2005), the spleen had already started to show accumulation of HbV 4 h after resuscitation. The lung and kidney did not show any abnormalities such as embolism in the capillaries derived from the aggregation of vesicles (Rudolph et al. 1995). In the chronic phase study, Hct showed complete recovery to the baseline 7 days after resuscitation, although difference between SAB group remained significant until two months after experiment. While HbV have disappeared from circulating blood by 7 days after infusion because the circulation half-life of HbV is about 35 h, phagocytized HbV might made effect on the delay of erythropoiesis recovery. In contrast, WBC and Platelet didn't show any significant differences between SAB and HbV/rHSA groups. This fact showed that HbV didn't make an influence on the kinetics of WBC and platelet.

Hemorrhagic shock and resuscitation induce ischemia, hypoxia, and reperfusion injury, all of which influence organ functions. Many precedent papers have described the elevation of plasma enzyme levels, such as AST, ALT, and LDH after resuscitation with HBOCs or transfusion (Sakai et al. 2004c; Marks et al. 1987; Lehnert et al. 2003; Bosman et al. 1992; Mota-Filipe et al. 1999; McDonald et al. 2002; Young et al. 2007). In the present study, the elevations of these plasma enzyme levels were also seen for the SAB group, and these are the common reaction for this kind of shock study.

Lipase activity, but not amylase, significantly decreased in the HbV group, whereas no histopathological abnormality was seen in the pancreas. In our previous tests of daily repeated infusion for 14 days or bolus injection using rats, a transient increase in lipase activity was observed. This was thought to be due to the up regulation of lipase in response to the infusion of a large amount of lipids from the liposomes (Stuecklin-Utsch et al. 2002). However, in this study, the result was in conflict with the past results. The reason is not clear, but the difference of species might be one possible reason.

Liposome-encapsulated Hb without PEG-modification aggregated in the plasma and showed a slight accumulation in the kidneys (Rudolph et al. 1995). However, our PEG-modified HbV does not induce intervesicular aggregation, and does not have any deteriorating influence on the kidneys. In this study, no abnormal value was noted for BUN, Cre, and UA and there was no histopathological abnormality in the kidneys in the HbV/rHSA group.

The plasma lipid components; T-Chol, F-Chol, and Total lipid significantly increased after the infusion in HbV/rHSA group. They should be derived from HbV because it contains a large amount of cholesterol, and they would be liberated

after the HbV particles are captured and degraded in the reticuloendothelial system (RES) (Sakai et al. 2001, 2004a). Extensive studies of circulation kinetics and organ distribution of isotope-labeled HbV clarified that HbV accumulates preferentially in the RES (Awasthi et al. 2004; Sou et al. 2005). It is reported that once liposome is captured in the Kupffer cells, the diacylphosphatidylcholine is metabolized and is reused as a cell membrane component or excreted in the bile (Dijkstra et al. 1985). Cholesterol is finally catabolized as bile acids in the parenchymal hepatocytes. There should be no direct contact of HbV and the hepatocyte because HbV (diameter, 250 nm) cannot diffuse across the fenestrated endothelium into the space of Disse (Goda et al. 1998). Cholesterol of the vesicles should reappear in the blood mainly as lipoprotein cholesterol after entrapment in the Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein cholesterol by the hepatocytes (Kuipers et al. 1986). Actually, it was confirmed that $^{3}H$ labeled cholesterol was excreted in feces by the experiment of rat (Taguchi et al. 2009). In terms of PEG-lipid, we reported previously that PEG chain disappeared within 14 days in the liver and spleen by the experiment of rat (Sakai et al. 2009). In the present study, the plasma lipid components increased until 7 days, and recovered at 14 days, and so, it is supposed that the lipid components of HbV would be completely metabolized within 14 days.

During the metabolism of Hb, we would expect a release of bilirubin and iron. But they did not increase in the plasma. The released heme from Hb in HbV could be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages (Sakai et al. 2004a; Finch and Huebers 1982). Bilirubin would normally be excreted in the bile as a normal pathway, and no obstruction or stasis of the bile should occur in the biliary tree. Normally, iron from a heme is stored in the ferritin molecule (Grady et al. 1989). Both ferritin and hemosiderin release iron. They are anticipated to induce hydroxyl radical production followed by lipid peroxidation (O'Connell et al. 1989). The iron release rate from hemosiderin, however, is substantially less than that from ferritin (Bennett and Kay 1981). Consequently, the excess amount of iron would then normally be stored in an insoluble and less toxic form as hemosiderin. We found iron deposit in the spleen and liver in long term study. The finding was the same with hemosiderosis often observed in patients who have received repeated blood transfusions.

The liver and the spleen are important organs for degradation of HbV in RES. Pathological examination of the liver showed evidence of Kupffer cells phagocytizing HbV; it disappeared within 7 days in the liver. In the spleen, substantial accumulation of HbV was confirmed in macrophages in the red pulp zone in the same manner as that in previous studies of bolus injection, daily repeated injections, and exchange transfusion (Sakai et al. 2001, 2004b, c). In the present study, hemosiderin deposition was detected in the liver of the HbV/rHSA group at 28 days. These results indicate that heme was metabolized in Kupffer cells of the liver and does not indicate a disability of liver function, as supported by the normal plasma enzyme levels.

We investigated lung, heart, liver, spleen, pancreas, kidney, adrenal glands, testis, trachea, esophagus, small intestine, and colon. Throughout the pathologic survey, we found no fibrotic change in organs. Perfluolochamical artificial oxygen carrier remained in the organs for over two years and induced fibrotic change in the liver (Kitazawa et al. 1982). Perfluorochamicals were said biological inert but it didn't have metabolic pathways. Therefore the material accumulate in the RES system and lymphnode. HbV were made of substantially biodegradable materials. Newly developed materials were minus charged lipid (DHSG) and PEG. Judging from the chemical structure, DHSG could be hydrolysed by non specific dehydrogenase, and the same pathways might be applied to PEG. We could not find any accumulation of these materials pathologically but further study was required.

In conclusion, resuscitation with HbV suspended in rHSA showed rapid recovery of hemodynamic parameters. There was no obvious side effect in hematological tests, plasma biochemical parameters, and histopathological examination within 1 year in comparison to the SAB group. Although transient but substantial accumulation of HbV in phagocytic cells raises concerns of the impact on the defensive function of the body, the present results using beagle dogs reassure that HbV suspended in rHSA shows a similar resuscitative ability and safety to that of SAB.

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# Part VII
# HBOC Clinical Trials

# Chapter 29
# Key Adverse Events in Recent HBOC Phase III Clinical Trials and Their Causal Relationship to Test HBOC's

**Colin F. Mackenzie**

## 29.1 Introduction

Alternative fluids for treating acute anemia may be required when blood and blood component therapy are refused or are not available, and tissue perfusion must be maintained. This chapter discusses the clinical trial population, side effects, toxicities and dosing of three hemoglobin based oxygen carrier (HBOC) solutions that have recently undergone Phase III clinical trials.

### *29.1.1 Clinical Trial Populations*

#### 29.1.1.1 HBOC's for Acute Anemia of Trauma

The primary initial resuscitation management of acute anemia caused by trauma includes decreasing blood and blood component loss from vascular injuries with rapid application of tourniquets, pressure bandages, surgical clamps and clotting gels until definitive vascular surgical control can be achieved (White et al. 2011; Starnes et al. 2006; Woodward et al. 2008). At the R Adams Cowley Shock Trauma Center (STC) of the University of Maryland, over 8,000 trauma patients are admitted each year. Approximately 2–3 % receive universal donor blood immediately on arrival, 3–5 % receive a massive packed red blood cell (pRBC)

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transfusion (variously defined as >4 units pRBC in 4 h, > 10 units pRBC in 6 h or >10 units pRBC in 24 h), and 8 % of STC trauma patients receive blood during their hospitalization with a 2–3 pRBC unit transfusion being the most common mode of administration (Como et al. 2004). There is therefore, currently a need for a universally compatible, intravenous oxygen-carrying solution in doses equivalent to 2–3 pRBC, with reduced risk of disease transmission to minimize multi-organ failure, and reduce mortality, This solution should have long-term storage capability for field and resuscitation use. Military battlefield casualties, (Moore et al. 2006) disaster scenarios, (Reid 2003) blood incompatibility, blood shortages, and religious objection (Mackenzie et al. 2010) represent additional situations in which a hemoglobin-based oxygen-carrying solution can address this medical need. Natural and man-made disasters, lack of a mature and widespread blood bank system in the majority of the developing world, and the threat of terrorism heighten the urgency for an alternative oxygen carrying solution to blood, that can allow survival until the casualty is transported to the available blood supply. There are 47,000,000 Americans who live more than 1 h from a trauma center, (Branas et al. 2005) and most ambulances do not carry blood (Gould et al. 2002).

Trauma patients with pre-hospital shock not receiving blood until arrival at the hospital have mortality rates of 17 to 54 % (Heckbert et al. 1998; Sloan et al. 1999; Mattox 1991; Bickell et al. 1994) and mortality increases with time and distance to definitive care. Rural settings account for only 20 % of the population but 60 % of trauma deaths because of longer transport times and lack of capabilities available in trauma centers, such as blood, during en route care (Landro 2007).

#### 29.1.1.2 Non Trauma Causes of Anemia and Treatment and Use of Blood or HBOC's

Acute surgical and medical anemia occurrence is usually non-elective and unanticipated, so blood may not be available, and HBOC's have a therapeutic opportunity. Management of anemia due to elective surgical and medical causes has interventions other than allogeneic blood transfusion, including pre-donation of autologous blood and anticipatory pharmacologic interventions with drugs such as iron folic acid and erythropoietin. Decreasing the transfusion requirement in all patients is important, because packed red blood cells (pRBC) are associated with infection, multi-organ failure, and mortality Claridge et al. 2002; Sauaia et al. 1994; Moore et al. 1997; Malone et al. 2003). In addition, stored pRBC sometimes cannot be used in the hospital because of medical causes such as inadequate inventory, acute immunologic states such as autoimmune hemolytic anemia, and certain religious groups will not accept transfusion (Mackenzie et al. 2010; Gould et al. 2002; Sloan et al. 1999). Finally, allogeneic pRBC transfusions may provoke adverse immuno-inflammatory responses in high-risk patients (Moore et al. 1997; Silliman et al. 2004).

### 29.1.2 Hemoglobin Based Oxygen Carrying Solution

The term *hemoglobin based oxygen carrying solution* includes blood (fresh, stored, frozen and lyophilized) and cell free hemoglobin based oxygen carriers (HBOC), with or without encapsulation in liposomes. Genetically produced HBOC have research interest as a tool to probe toxicity, but their clinical use is currently restricted by their cost and inability to produce large quantities sufficient to provide supplies for an appropriately powered clinical trial. HBOC functions include: carrying and delivering oxygen and augmenting blood volume.

### 29.1.3 Rationale for HBOC Use

More than two-thirds of the world does not have adequate blood supplies (Klein et al. 2007). There may be a lack or shortage of compatible blood, the means to collect and process blood, and the means to keep blood refrigerated and have it available at all times for all those requiring transfusion. Blood shortages are expected to occur during mass casualty events, military operations, natural disasters, or terrorist attacks. HBOC lack the numerous and complex antigens of the pRBC membrane, so are universally compatible, and can be readily administered to patients in shock. HBOC's universal donor status (due to removal of red cell wall antibodies in processing) avoids the need for cross-matching. The ambient temperature (2–30 °C) stability of HBOC-201 (Hemopure, OPK Biotech LLC, Cambridge, MA), its prolonged shelf life of 3 years, and its equivalent hemoglobin concentration to whole blood, make HBOC-201 an ideal choice for field use and for stockpiling, especially in rural areas distant from blood bank facilities. When compatible blood is not available or needs to be conserved due to recipient rare red-cell types, HBOC can function as a fluid for intentional hemodilution to conserve red cells, or can be used in tandem with other conservation techniques under the rubric of 'bloodless surgery'. In addition, HBOC have benefits in extending the useful life of donor organs awaiting transplant.

Non-blood HBOC fluids have no cells, so the rheology in acute anemia management is optimized, and oxygen delivery can be enhanced to reverse ischemia in tissues beyond arteriolar stenosis, and inaccessible to red cells (Weiskopf 2011). Concern about infectious and immunosuppressive risks of allogeneic blood, and current convergence of blood quantities donated in comparison to those consumed, makes HBOC a practicable means of supporting demand in the face of diminishing blood supply (Zou et al. 2008; Vamvakas and Taswell 1994).

## 29.2 Cell Free Hemoglobin Based Oxygen Carriers and Known Toxicities

Essential biochemical, physiological and logistical characteristics of the three HBOC that have undergone Phase III clinical trials in comparison to pRBC are listed in Table 29.1. The cell wall of the red blood cell limits the potential toxicity of the free hemoglobin within, by preventing interactions with the endothelium and plasma components of blood. Potential reactions of free hemoglobin binding to endothelial and smooth muscle nitric oxide (NO) receptors are avoided by the red cell wall, that also prevents hemoglobin/tissue oxidative reactions causing oxidation of globin proteins and lipids, and hemin/protein interactions that cause aggregates (Silverman and Weiskopf 2009). Free hemoglobin can occur outside the red cell in many clinical situations (Table 29.2). The general schema for possible causes of free hemoglobin toxicity is summarized in Fig. 29.1.

The development of HBOC's has increased the awareness of their synergistic effects on the vasculature at cellular and functional levels. However, there is no consensus on the underlying mechanisms of the toxicities of HBOC that have undergone Phase III clinical trials including HBOC-201, MP4OX, and Polyheme, in part because these modified hemoglobin solutions are very different in physical terms, such as molecular size, surface properties and also in physiological, biochemical, pharmacological properties (Bettati 2011). So a 'one size fits all' mechanistic model for their toxicities is unlikely.

**Table 29.1** Comparison of HBOC's in clinical trials with red cells

| Feature | Hemopure/ HBOC-201 (OPK biotech) | PolyHeme (PolySFH-P) Northfield laboratories inc. | Hemospan/ MP4 (Sangart) | Red blood cells |
|---|---|---|---|---|
| Hemoglobin source | Bovine | Human | Human | Human |
| Type of modification | Polymerization | Glutaraldehyde polymerization | Polyethylene glycol conjugation | Not applicable |
| Average molecular weight (kDa) | 64–500 | 150 | 90 | Not applicable |
| Hemoglobin level (g/dl) | 13 | 10 | 4.2 | 13 |
| Volume (ml) | 250 | 500 | 250 or 500 | |
| $O_2$ pressure at 50 % oxygen saturation (mmHg) | 40 | 26–32 | 5–6 | 26–27 |
| Oncotic pressure (mmHg) | 25 | 23 | 49 | 25 |
| Viscosity (cP) | 1.3 | 2.1 | 2.2–2.5 | 5–10 |
| Half-life | 19 h | 24 h | 43–66 h | 31 days |
| Shelf life | <3 yrs (at 2–30 °C) | <1 yr (at 2–8 °C) | <3 yrs (frozen) | 42 days (at 4 °C) <6 h (at 21 °C) |

**Table 29.2** Clinical causes of free hemoglobin

| |
|---|
| "Leakage" from transfused stored red blood cells |
| Transfusion reactions |
| Sepsis, myocardial infarction, and stroke |
| Acute hemolytic disorders such as sickle cell disease and autoimmune hemolytic anemia |
| Hemolysis secondary to drug interactions |
| Trauma to the red cell (e.g. roller pump causing leakage of hemoglobin) |
| Administration of hemoglobin based oxygen carrying (HBOC) solutions |

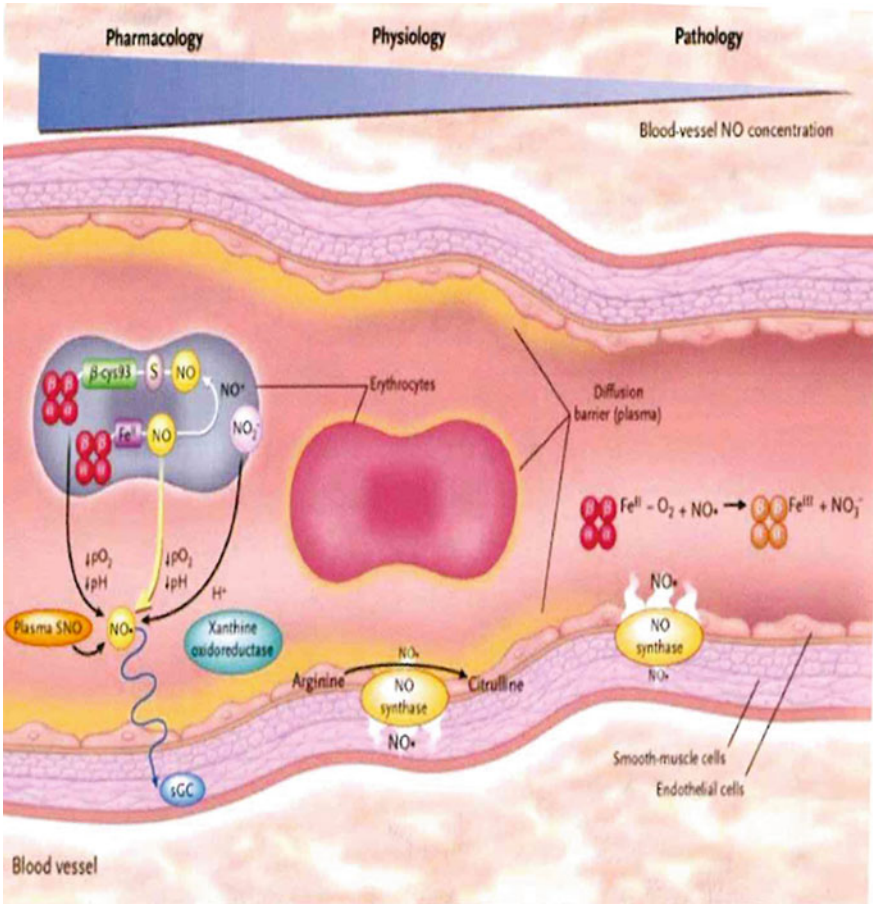


**Fig. 29.1** A model of the interactions between nitric oxide (NO) with erythrocytes and cell free HBOC in an arterial blood vessel showing the processes involved in gaseous or NO donor delivery of NO on *left* side, the normal state when hemoglobin is within the red cell wall, and in abnormal state of HBOC infusion or pathological state of free hemoglobin leakage from within the red cell. Blood vessel NO concentration is shown as the blue bar above the vessel. Free hemoglobin binds NO resulting in vasoconstriction of the vessel to *right* of the diagram (Reprinted with permission of Massachusetts Medical Society from Schechter AN, Gladwin MT. Hemoglobin and the paracrine and endocrine function on nitric oxide. N Engl J Med 348: 1483–1485 Figure 1.)

Current HBOC elicit serious adverse events (as defined by FDA) including elevated blood pressure, myocardial infarction, stroke, hemostatic effects (platelet aggregation) and renal toxicity (Silverman and Weiskopf 2009). However, risks are not uniform across all populations. Probable differences occur by patient age, disease process, and other important factors. Toxicities are likely to be exacerbated by pre-existing vascular dysfunction including hypertension, diabetes and obesity. It is possible that populations of improved benefit-to-risk ratio for HBOC use include younger age and minimal co-morbidities (Freilich et al. 2009).

One of the major challenges facing the development of HBOCs is minimizing their vasoactive properties. Soluble hemoglobin, unlike hemoglobin in RBCs, interacts with nitric oxide (NO) to form methemoglobin and S-nitroso hemoglobin (by interaction with heme thiol) and nitrosyl hemoglobin (via interaction with heme iron). NO is a potent endothelial vasorelaxant that inhibits the conversion of pro endothelin to the vasoconstrictor endothelin. In the prevailing theory on vasoactive properties, NO sequestration by hemoglobin is responsible for vasoconstriction (McMahon 2011). Impaired blood flow in the presence of vasoconstriction can cause greater endothelial interaction, hyper oxygenation and impaired vascular regulatory processes such that eNOS becomes uncoupled and generates superoxide rather than NO. The rate of nitric oxide scavenging can be reduced by modifying free hemoglobins to decrease the HBOC-induced increase of blood pressure. Alternative theories suggest that too much oxygen is delivered causing an autoregulatory vasoconstrictor reflex. However, this excess oxygen mechanism does not explain pulmonary hypertension occurring with administration of some HBOC's, as oxygen is a pulmonary vasodilator, meaning that another mechanism is active. Yet another theory argues that oxidation of soluble hemoglobin can result in heme loss, free radical formation, loss of reactive iron, and oxidation of lipids. Such reaction and products result in endothelial stress causing vasoconstriction (Alayash 1999), but using protein engineering, HBOC's can be developed with resistance to auto and chemically-induced oxidation and rate of heme loss and the effects of such HBOC's need to be tested.

A recent meta-analysis concluded that there was no role for any of the HBOCs currently in clinical development and proposed that a "HBOC class effect" is responsible for myocardial infarction and increased mortality (Natanson et al. 2008). However, the meta-analysis is seriously flawed, and the statistical tools and methods used inappropriately combined dissimilar studies, as described in a series of letters to the Editor (Levien et al. 2008; Keipert et al. 2008; Shander et al. 2008; Sauaia et al. 2008). The proposed HBOC cardiotoxic 'class effect' appears unlikely with such a diversity of biochemical, physiological and surface coatings and molecular size. Myocardial infarction appears unlikely to be a 'class effect' for HBOC-201 (Fig. 29.2) as continuous infusion reduced troponin levels dramatically in humans with both normal and abnormal myocardial perfusion and in animals with induced myocardial ischemia as well as ischemia rescue and applications in cardiology and vascular surgery (Te Lintel Hekkert 2010; Meliga 2008; Fitzgerald et al. 2011). Statistical significance for mortality 'class effect' in the meta-analysis derives solely from one HBOC (Hemassist), withdrawn some

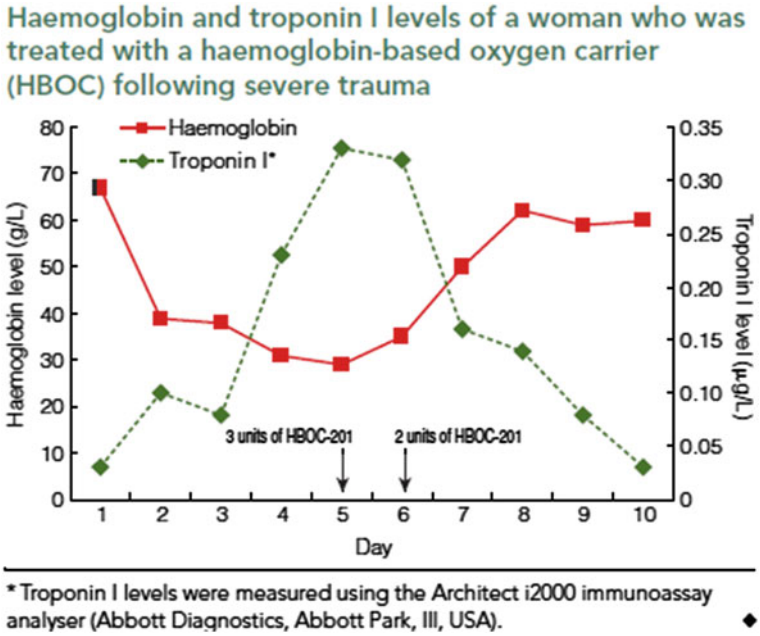


**Fig. 29.2** Hb and troponin I (Abbott Architect i2000) levels during the first 9 days of admission (used with permission from Fitzgerald MJ et al. Med J Aust 2011; 194: 471–473). Reproduced with permission from: Galvagno SM, Mackenzie CF. New and future resuscitation fluids for trauma patients using hemoglobin solutions and Hypertonc saline. Anesthesiology Clin 31 (2013) 1–19

15 years earlier. Meta-analysis methodology evaluates effects of multiple trials, and does not speculate on mechanism of action or the risk-to-benefit ratio of any single product. The risk-to-benefit ratio of such a clinical situation in which blood

**Table 29.3** HBOC benefits over blood

| |
|---|
| Ready to use with no planning, and no equipment, blood collection or blood processing laboratories and cost |
| No donor recruitment, no collection (personnel/equipment), no blood transport and distribution |
| No Pre-transfusion prep, limited blood hemovigilance and paperwork |
| No processing and separation of components |
| No waste as HBOC 30–50 times longer than blood until expiration date. Long HBOC shelf life, allows stockpiling for battlefield readiness, disasters, managing post partum hemorrhage in remote areas, in EMS ambulances and helicopters |
| Some HBOC need no refrigeration, hence availability in 2/3rd of Developing world with inadequate blood bank facilities |
| Universally compatible, could replace un-cross matched blood use. No transfusion errors as no cross-match |
| Immediately offloads oxygen, no 2, 3-DPG dependant tissue oxygen off-loading |
| May be used by Jehovah's Witnesses |
| Provides oxygenation when blood not an option e.g. Auto-Immune Hemolytic Anemia |
| Extends the useful life of donor organs awaiting transplant |

is limited, not available or not an option, must weigh the risks associated with HBOC against the risk of death (Mackenzie 2009; Galvagno and Mackenzie 2013). The benefits of HBOC over pRBC are summarized in Table 29.3.

## 29.3 HBOC Safety and Efficacy

### *29.3.1 HBOC-201 (Hemopure)*

HBOC-201 (Hemopure) is approved by Regulatory Authorities for human use in South Africa and in Russia, and was used in the largest Phase III clinical trial (HEM-0115) of any HBOC (Jahr et al. 2008) (n = 350 subjects randomized to HBOC-201) and has been administered to more than 800 humans in 22 completed clinical trials including four pRBC controlled trials in cardiac, vascular, general non cardiac, and orthopedic surgery, (Gawryl et al. 2006) and for compassionate use in more than 70 patients up to September 2012 (Mackenzie et al. 2010). HBOC-201 is a polymerized iso-oncotic high molecular weight HBOC of bovine origin. Transfusion sparing properties of HBOC-201 were demonstrated. HBOC-201 is free from pro-inflammatory stimuli and does not transmit disease. HBOC-201 has not received approval by the US Food and Drug Administration (FDA) other than under 21 CFR Parts 312 and 316: (Expanded access to investigational drugs for treatment use) (Food and Drug Administration 2009). The FDA cited serious adverse events causing failure to obtain approval after the HEM-0115 trial (see below).

These adverse events resulted from a combination of compromised elderly patient co-morbidities, and various clinical mis-management issues including initial hemodilution with crystalloids, followed by over-infusing HBOC-201 with resulting heart failure. In addition, there was a failure to maintain plasma hemoglobin levels consistent with the 19 h half-life of HBOC-201. Half-life of pRBC is 22–33 days, so management of acute anemia is more readily sustained. Total hemoglobin concentration in HEM-0115 patients randomized to HBOC-201 (containing 13 g/dl hemoglobin) was lower than in patients randomized to pRBC (containing 32–36 g/dl of hemoglobin), affecting overall efficacy and safety results (Jahr et al. 2008). Because patients randomized to HBOC-201 were maintained at a lower hemoglobin than those randomized to pRBCs, HBOC patients had a persistent anemia and lower on-going oxygen carrying capacity that did not exist in patients randomized to pRBC. Total hemoglobin concentration in patients randomized to HBOC-201 versus pRBC was less, which affected overall FDA efficacy and safety results. The mortality of those randomized to HBOC-210 in the HEM-0115 trial was higher (10 vs. 6 in pRBC group) although the independent safety evaluation committee did not relate mortality in either group to treatment. In those patients over 80 years old there was a mortality imbalance (16.1 % in HBOC-201 vs. 3.9 % in pRBC), possibly related to the persistent anemia (Jahr et al. 2011).

If, alternatively to the persistent anemia, an intrinsic toxicity was responsible for the safety imbalance, a positive correlation between adverse events and increasing dose would be expected in only the HBOC group, which as Figs. 29.3 and 29.4 shows, was not the case. Despite this hemoglobin concentration difference, there was a 96 % transfusion avoidance for 24 h in those randomized to HBOC-201, allowing adequate time to sustain field resuscitation, and to transport patients to facilities with blood for transfusion. There were no adverse event or serious adverse event differences between those receiving $\leq$3 pRBC or equivalent amounts of HBOC-201 (Figs. 29.3 and 29.4). Within one week of HBOC adminstration, there was 67 % transfusion avoidance, making HBOC-201 a practical alternative when blood is unlikely ever to become available, because erythropoesis due to the iron load after HBOC-201 infusion, restores total hemoglobin concentration within 7-10 days. A further analysis of HEM-0115 data showed an acceptable safety profile in younger acute trauma populations, especially in settings where rapid access to safe blood transfusions is unavailable (Silverman and Weiskopf 2009). HBOC-201 is metabolized through the liver and reticulo-endothelial system (Beuzard et al. 1995) so renal toxicity is minimal. A controlled clinical trial (HEM-0114) showed transfusion sparing, but no differences in AE's and SAE's, mortality or morbidity outcomes in doses up to 7 units HBOC-201 in 6 days compared to pRBC for elective non cardiac surgery (Personal Communication Z Zafirelis Oct. 4th 2012). In South Africa there have been no issues regarding management of blood pressure, myocardial infarction, renal effects, mortality and adverse events/serious adverse events in over 480 patients (including trauma and elective surgery patients) treated with HBOC-201 (Levien 2006). A Pre-Hospital trauma patient resuscitation clinical trial with HBOC-201, has received regulatory and ethical approval to begin shortly in Victoria, Australia. The trial design and setting differs from the Polyheme trial (see below) in that there are longer pre-hospital transport times, and the trial includes more robust Shock Index =/> 1 enrollment criteria, younger patients (<55 years of age), administration of HBOC-201 ceases on trauma center admission.

MP4 OX (MalPEG-hemoglobin) is a polyethylene glycol (PEG)-conjugated hemoglobin, and is being tested as a volume expander with oxygen carrying capability. MP4OX has chemically cross-linked human hemoglobin chains resulting in molecules 128 kDa or larger that are not readily filtered by the glomerulus, thus greatly increasing half-life. MP4OX has a low hemoglobin concentration, requires refrigeration, cannot be stored for prolonged periods at higher than room temperature, and is not approved for human use by any regulatory body. This molecule is characterized by high oxygen affinity that limits oxygen release in arterioles and has decreased rates of oxidation, with low hemoglobin concentration and increased oncotic pressure. These characteristics suggest that MP4OX might preserve functional capillary density in anemic and shock states and reduce the incidence of hypertension and other adverse events. Six clinical trials in elective surgery, chronic critical limb ischemia, and prevention of hypotension are either underway or already completed. Safety results of a Phase II trial in orthopedic surgery (Vandergriff et al. 2008; Winslow 2007; Olofsson et al. 2006; Olofsson

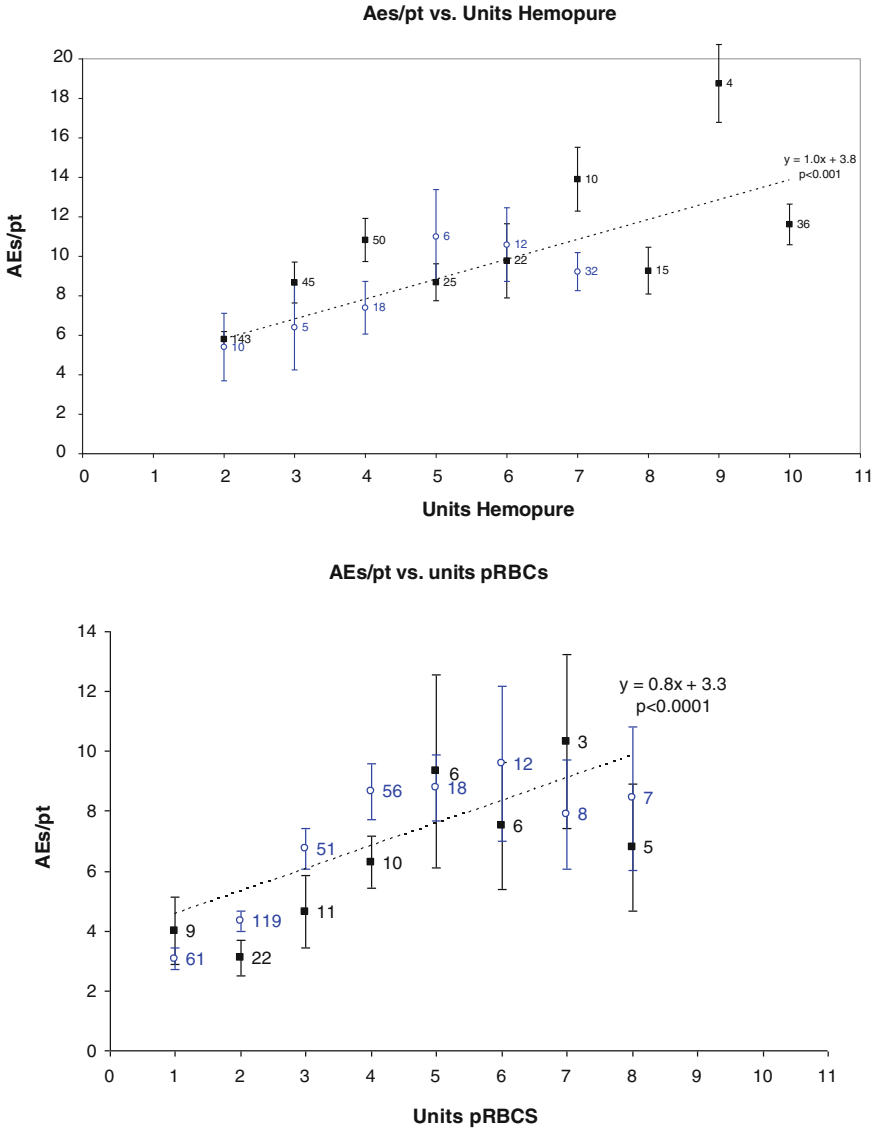


**Fig. 29.3** *Upper panel* regression analysis of the relationship between adverse events (AEs)/patient (pt) tand dose (*in units*) of Hemopure received in study HEM-0115 (*black filled squares*) and study HEM-0114 (*blue open circles*). *Lower Panel* AEs/pt as a function of Dose of pRBCs: Regression analysis of the relationship between AEs/pt and dose (in units) of pRBCs received in study HEM-0115 (*black filled squares*) and study HEM-0114 (*blue open circles*). The correlation between the dose of pRBCs and incidence of AEs in an acutely anemic population is driven by the patient's clinical need and the patient's underlying co-morbidities. Similar correlations are observed between increasing doses of pRBCs or Hemopure and increasing rates of FDA Defined AEs/patient. Reproduced with permission from: Galvagno SM, Mackenzie CF. New and future resuscitation fluids for trauma patients using hemoglobin solutions and hypertonc saline. *Anesthesiology Clin* 31 (2013) 1–19

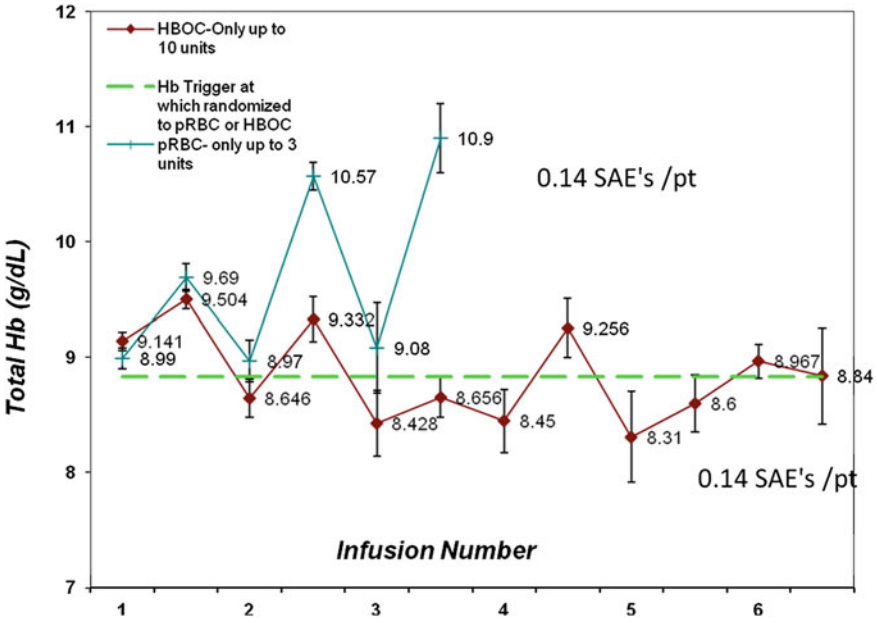


**Fig. 29.4** Effects of dosing HBOC-201 alone up to 10 units and pRBC alone up to 3 units on total hemoglobin (Hb) in HEM-0115 trial. Incidence of Serious Adverse Events/patient (pt) was 0.14/pt in both groups. Mann–Whitney estimator of safety non-inferiority (MW), derived from results of an independent review of each case report and medical records by at least two physician reviewers of a 27 member independent Safety Endpoint Evaluation Committee. MW = 0.519 95 % CI 0.481–0.558. Reproduced with permission from: Galvagno SM, Mackenzie CF. New and Future Resuscitation Fluids for Trauma Patients using Hemoglobin Solutions and Hypertonc Saline. *Anesthesiology Clin* 31 (2013) 1–19

et al. 2008; Linden et al. 2011; Olofsson et al. 2011) showed an imbalance in cardiac and vascular events, including bradycardia and blood pressure elevation, gastrointestinal events including nausea, cardiac rhythm changes, and pancreatic enzymatic activity (lipase and amylase) with greater frequency after administration of MP4OX than placebo (Ringer's acetate). The small increases in mean arterial pressure seen in clinical trials may be attributable to MP4OX's hyperoncotic volume-expansion properties rather than nitric oxide scavenging. In these dose escalation clinical trials, plasma oxygen content was significantly increased even with modest doses of MP4OX, suggesting that increased oxygen delivery could be obtained.

Current testing (http://clinicaltrials.gov/ct/show/NCT00420277?order=1) will determine if MP4OX can improve perfusion and oxygenation of ischemic tissues in trauma patients in hemorrhagic shock with lactic acidosis (serum lactate level $> -5$ mmol/L) within 2 h of arrival at the study hospital and within 4 h of traumatic injury. Two dose levels 250 and 500 ml are being compared to isotonic

Ringer's lactate solution in reducing lactic acidosis. Secondary outcomes include, SOFA score, renal replacement, vasopressor use. Adverse effects and mortality outcome will be evaluated through day 28.

Polyheme, a gluteraldehyde polymerized human hemoglobin with very low amount of tetramer, successfully used in management of trauma patients and those refusing pRBC transfusion (Gould et al. 2002). Polyheme completed a Phase III clinical trial using administration in a setting of prehospital and initial 12 h of hospitalization, to patients with major trauma. Polyheme was compared to standard care of crystalloid in the field and pRBC on hospital arrival. In total 720 patients (48 % blunt Vs. 52 % penetrating trauma) with systolic blood pressure $<-90$ mmHg were randomized. Patients received up to six units (50 g/dl hemoglobin/unit) beginning at the scene and continuing during the first 12 h after injury, after which pRBC were given if needed (Jahr et al. 2011).

Polyheme failed to achieve its dual primary end point of superiority/non-inferiority of mortality outcome (compared with expected mortality in a rural setting with longer transit times to hospital and availability of blood). Thirty day mortality (13.4 % Polyheme and 9.6 % control).

And 30 days mortality for injury sub-groups were significantly higher in those randomized to Polyheme (Silverman and Weiskopf 2009). In total six clinical trials in 1,133 subjects, 674 of whom received PolyHeme have been conducted. The pre—hospital Phase III study was designed to assess the benefit of Polyheme to the intended population for whom blood would not be available for prolonged periods of time; it was not designed to demonstrate that Polyheme could be used in place of blood (19). Mortality assessments included the Intent-to-Treat (ITT; as randomized), As-Treated (AT; as treatment received), and Per Protocol (PP; without major protocol violations) populations were presented. In the ITT population, the mortality was 13 % (47 of 350) and 10 % (35 of 364) for the control group, failing the prespecified 7 % non-inferiority boundary. An as per protocol analysis showed that subjects randomized to PolyHeme had on average lower blood pressure before randomization and more severe neurologic findings, more severe coagulopathy at randomization, and more severe injuries than subjects in the control arm (Silverman and Weiskopf 2009; Jahr et al. 2011).

Serious adverse events were reported for 40 % (141 of 349) test subjects and 35 % (126 of 365) control subjects. The most common serious adverse events that occurred in excess in test subjects included pneumonia, multiple organ failure, hemorrhagic shock, respiratory failure, hypercoagulable state, coagulopathy, and myocardial infarction (3 % in those randomized to Polyheme vs. 1 % in the control group). Polyheme had no known renal toxicity. A blinded assessment of cardiac events by experts of the data monitoring committee found no difference between treatment groups in this study and suggested that myocardial infarction in victims of trauma is much more common overall than previously believed (Silverman and Weiskopf 2009; Jahr et al. 2011). However the disproportionate mortality lead to the FDA application for Polyheme failing to be approved, resulting in closure of Northfield, the manufacturer.

## 29.4 General Side Effects Associated with HBOC's and their Mitigation

Clinical affects noticed in association with HBOC administration include, gastrointestinal effects, (nausea, vomiting, diarrhea, abdominal pain and bloating). Binding of nitrous oxide to gastrointestinal intestine tissues is the proposed cause. Skin rashes, jaundice without elevated bilirubin (pre-hepatic jaundice based on increased bilirubin load consistent with physiological processing of HBOC-201), fever, and interference with laboratory assays due to high concentrations of plasma hemoglobin causing anomalies such as elevation in lipase levels and interference with tests for liver enzymes, bilirubin, amylase. Inaccurate results in the presence of HBOCs may be avoided by use of different analytic devices. Clinicians should be aware that methemoglobinemia can be a problem, especially at low red cell hemoglobin concentration, when methemoglobin reductase (in the red cell wall) levels are low. Side-effects can be mitigated (e.g. by nitric oxide donor, beta blocker or calcium channel blocker or changing infusion rate for blood pressure, use of diuretics to reduce volume effects, methylene blue infusion or use of ascorbic acid to reduce methemoglobin, and anticholinergics for gastrointestinal discomfort) (Galvagno and Mackenzie 2013).

## 29.5 Dosing and Monitoring of HBOC Infusion

Intravenous dosing of HBOC should be monitored with total hemoglobin concentration not hematocrit (as HBOC are acellular), where total hemoglobin = red cell + plasma hemoglobin (from HBOC) and total hemoglobin should be maintained at least >5 g/dl by continuous slow (2–6 h) infusion of consecutive units of HBOC. Rate of infusion may have an effect on the incidence of toxicity. Continuous infusion may saturate endothelial nitric oxide binding, stabilizing vasoconstriction, whereas intermittent bolus infusions could allow interval recovery of nitric oxide induced vasoactivity. Re-dosing should not wait until the plasma half-life (12–36 h for the three HBOC's) is exceeded, rather HBOC should be continuously infused to ensure therapeutic effect and maintenance of oxygen carriage (Galvagno and Mackenzie 2013).

## 29.6 Conclusion

Stabilization of trauma patients in shock requires aggressive hemorrhage control and restoration of tissue perfusion. HBOC have both physiological and logistical advantages over standard isotonic crystalloids to restore tissue perfusion, to bridge critical gaps when blood and blood component therapy is not available, buying

time to facilitate definitive surgical hemorrhage control. From the three HBOC discussed, toxicities are not a 'class effect' , they are generally thought to be due to binding of nitric oxide by free hemoglobin, so may be mitigated by simple interventions to minimize endothelial stress allowing the potential toxicity risks to be outweighed by the obvious clinical logistical benefits. HBOC could be useful in the prehospital environment, military conflicts, disasters, and in the developing world, and in other situations where blood is not readily accessible. HBOC could potentially replace the standard 2–3 unit pRBC transfusions that 20–35 % of all transfused patients admitted to trauma centers receive (Como et al. 2004). HBOC should be considered for the reversal of acute anemia in circumstances when blood is not available or is refused. The risk/benefit of HBOC in comparison to exsanguination or persistent severe anemia is in favor of HBOC, as HBOC toxicities are transient, readily reversible, and less than 1 % result in fatalities.

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# Chapter 30
# Some Critical Comments on the Major HBOC Clinical Trials

**George P. Biro**

*The only thing potentially worse than not being able to see the forest for the trees is not being able to see the trees because of the forest.*

Anonymous

## 30.1 Objective

The objective of this chapter is to offer some critical comments on the major HBOC clinical trials, to follow the earlier chapters in this volume describing in detail the trials completed on the various products. Of necessity, the comments to follow will emphasize some of the negative aspects of the trials that may have accounted for their failures to satisfy the regulatory authorities to issue licenses for any of the products, except for the Republic of South Africa. The author has been an executive at Hemosol Inc. and was an active participant in the Company's clinical trials management. The comments to follow represent reflections from this experience and are solely the opinions of the author.

## 30.2 Introduction

A total of seventeen published reports are available on four HBOC products of major (Phase II and III) clinical trials completed by commercial developers. In these trials the HBOC product was compared against current therapies for hemorrhagic hypovolemia in acute trauma in emergency settings and bleeding in elective surgery settings, including coronary artery bypass grafting (CABG), mixed non-cardiac, aortic and orthopedic surgery. In addition to the published reports some reviews are also available (Vlahakes 2000; Greenburg and Kim 2004; Chen et al. 2009; Silverman and Weiskopf 2009; Weiskopf 2010; Elmer et al. 2012).

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*In emergency settings* the trials included resuscitation using HBOC either in the emergency department (Sloan et al. 1999b), or pre-hospital, in the ambulance (Kerner et al. 2003), sponsored by Baxter Healthcare Corporation, using their Diaspirin Cross Linked Hemoglobin (DCLHb) product HemAssist ®. The total number of subjects exposed to DCLHb in the two emergency trials was 141. In the in-hospital trial, conducted at 17 trauma centers in the USA, DCLHb was infused in the emergency room in a volume not exceeding 1000 mL within the first 2 h of arrival. The study at termination comprised 52 HBOC and 46 control subjects infused with HBOC or saline, respectively. The parallel pre-hospital on-scene trial was conducted in 27 centers in three European countries. Those eligible subjects who were randomized to receive DCLHb were infused with up to 1,000 mL in the ambulance. Fifty-eight subjects actually received DCLHb and 63 control subjects received the standard treatments used locally, including fluids, vasopressors, blood, etc. Both studies were halted before the originally planned number of subjects had been enrolled.

Another commercial developer, Northfield Laboratories Inc, conducted the first Phase III trial in acute traumatic hemorrhagic hypotension and emergent surgery, using their hemoglobin product, PolyHeme® at 29 US trauma centers. The trial was intended to demonstrate the utility of the product in "simulated unavailability" of blood as the first medium of resuscitation in the field and during the first six post-injury hours. Three hundred and fifty subjects were enrolled in the treatment arm and 364 in the control arm (Moore et al. 2009).

BioPure Corporation and the US Navy applied to conduct a trauma trial in the emergency setting (RESUS) but regulatory approval was withheld based on the unfavorable safety profile of the product in non-emergency elective surgical patients.

*In elective surgery setting* Baxter Healthcare Corp. sponsored Phase II and Phase III trials in cardiac surgery (Lamy et al. 2000a), elective aortic surgery (Bloomfield et al. 2004) and in elective non-cardiac surgery (Schubert et al. 2003). A total of 201 subjects were exposed to DCLHb in doses ranging up to three units of 250 mL each.

BioPure Corporation sponsored Phase II and Phase III trials in orthopedic surgery (Jahr et al. 2008; Freilich et al. 2009), aortic surgery (LaMuraglia et al. 2000), general surgery (Sprung et al. 2002) and cardiac surgery (Levy et al. 2002). In these trials a total of 457 subjects were exposed to HemoPure® in doses potentially up to a total of 10 units of 32.5 g hemoglobin. An additional 12 subjects undergoing elective aortic repair were exposed to 1 L of HemoPure® without comparison to transfusion to collect oxygen transport and hemodynamic data only (Kasper et al. 1998).

Hemosol Corp. sponsored two Phase III trials in cardiac surgery but the results of only the first trial were published "in full"(Hill et al. 2002; Greenburg et al. 2004). The subsequent trial was halted because of an excess of cardiac adverse events that, it should be noted, occur commonly in cardiac surgical patients.

## 30.3 Trial Endpoints

### *30.3.1 Efficacy Endpoints*

#### 30.3.1.1 Emergency Trials

The *primary* endpoint in these trials was improved survival to 28 or 30 days post-injury compared between HBOC treated and pRBC (packed red blood cells) treated subjects (Gould et al. 1998; Sloan et al. 1999a) and the number of organ failures (Kerner et al. 2003), as well as in the Northfield trauma trial (Sloan 2003) .

Randomized controlled trials in the traumatic hemorrhagic shock environment with a survival end point are difficult and fraught with methodological and conceptual problems. A systematic review of 11,848 published trials found only 35 to be susceptible to qualitative analysis (Curry et al. 2011). Only one of these comparative blinded trials succeeded to find a significant advantage for the test article, trenaxemic acid. There was also significant diversity in enrollment criteria based on a threshold systolic blood pressure as the definition of hemorrhagic shock (range from 80 to 100 mmHg). Of necessity, such trials enroll subjects with a wide range of age, nature and severity of trauma and magnitude of blood loss. Whereas Injury Severity Score (ISS) data were collected in HBOC emergency trials and for the most part, the *mean* scores were similar between the treatment arms, but this does not guarantee that the treatment and control arms were fully comparable in all aspects that contribute to the probability of late survival either to hospital discharge or to 28 or 30 days post-injury. This was demonstrated *post hoc* in the Baxter trauma trial in that estimated mortality risk was found to be biphasic, rather than normally distributed (Sloan 2003). Hence, those most severely injured would not benefit from any postulated benefit attributable to the HBOC. Moreover, the standard of care for the post-injury resuscitation of the severely injured, as of the time of this writing, is still not settled (Wagner 2011) and variations in clinical practice abound. Hence, significant uncertainties complicate conclusions drawn from these complex and difficult trials.

Sloan (2003), in comparing the two trauma trials sponsored by Baxter Healthcare noted that significant differences emerged between the two trials after post hoc reviews. In the US trial HemAssist® recipients had a higher mortality than those in the control arm (47 % vs. 25 %, $p = 0.015$) but this difference was not seen in the European trial. Nonetheless, both trials were halted when only 14 % of the planned number of subjects ($n = 850$) had been enrolled. The two trials also differed in the duration of the initial period to treatment; in the European trial with very short ambulance response times blood loss was managed early and the principal eligibility criterion (systolic BP $< 90$ mmHg) was felt to be inappropriate. Lastly, and perhaps most importantly, post hoc review showed that a number of protocol violations occurred in the US trial by enrolling subjects clearly ineligible because they had suffered a traumatic arrest, severe head injury or had a base deficit >15 mEq/L, and that these protocol violations were more numerous in

the HBOC treated arm. Intent-to-treat analysis, pre-specified in the IND approval, included these subjects showing a greater mortality risk.

The trial of PolyHeme® sponsored by Northfield Laboratories on emergency trauma subjects treated either with up to six units of the HBOC ($n = 349$) or with crystalloid and allogeneic pRBC transfusion ($n = 365$) found no survival benefit at 28 days attributable to PolyHeme® (Moore et al. 2009). This trial also showed a substantial number of protocol violations (17 %) and an analysis of the "as-treated" sub-population also failed to meet the primary endpoint. It is possible that protocol violations may be unavoidable under the often desperate and critical conditions in trauma centers, but if protocol violations are unevenly distributed between treatments, their impact is difficult to estimate. This may be critical if the violations directly affect trial endpoints.

It may also be questioned that survival to 30 days of a seriously or critically injured subject may be affected by a large number of factors in addition to that of the adequacy of the initial resuscitation within the first 24–48 h. This endpoint however was recommended by an FDA Advisory Panel. It seems intuitive that the initial treatment whereby systemic and critical organ oxygenation is rapidly restored would have an important impact on the subsequent development of organ failure, sepsis and thus, on ultimate survival, but convincing evidence of this is lacking. In fact in the Baxter European trial the endpoint of the number of organ failures tried to address this link. In the Northfield trial the argument was advanced that allogeneic blood transfusion was associated with immune modulation and that bank blood may stimulate an inflammatory reaction, or exacerbate the systemic inflammatory reaction associated with trauma. This argument was based on preclinical and clinical evidence that suggested that PolyHeme® may attenuate the systemic inflammatory reaction and thereby the likelihood of subsequent organ failure (Moore et al. 2005, 2006). This contention, in the event, was not supported by the trial data. Northfield investigators further argued (Bernard et al. 2011), based on a post hoc analysis of the data, that the 30-day survival endpoint was not appropriate for the study because it was intended to test the efficacy of PolyHeme® not in level I trauma centers, but in the *absence* of early availability of red cell transfusion at a distance from definitive high-level care. The reanalyzed data demonstrating an early (first 24–48 h) survival advantage of PolyHeme® over crystalloid resuscitation and do support the potential application of an HBOC as a *temporary* resuscitation fluid until pRBC transfusion becomes available. The secondary question raised by the trial results of how to balance the risks of later mortality against the benefits of "effective" early resuscitation in the *absence* of available blood cannot be settled here.

#### 30.3.1.2 Elective Surgery Trials

Moore (2004) summarized the objectives of FDA-approved HBOC trials:

> Perioperative reduction of allogeneic RBC transfusion is one of the three areas identified by the Center for Biologics Evaluation and Research of the FDA to target for the clinical investigation of HBOCs. FDA authorized Phase III studies are designed primarily to establish efficacy while assuring safety in patients with more advanced disease. In the arena of biologic products, efficacy is defined as a reasonable expectation that the product will result in a direct benefit to the patient.

The primary endpoint in elective surgery trials was prevention or a significant reduction of allogeneic pRBC transfusions.

In all of the non-emergency, elective surgery trials (Lamy et al. 2000; Schubert et al. 2003; Bloomfield et al. 2004) supported by Baxter International in cardiac, non-cardiac and aortic surgeries some allogeneic blood sparing was reported with the use of DCLHb. Likewise, the trials sponsored by BioPure also showed some sparing of blood. In particular, in the elective cardiac surgical setting (Levy et al. 2002) one third of HBOC subjects *avoided* allogeneic rbc transfusion and those who did not, received *22 % less* in total volume than control subjects. In aortic surgery (LaMuraglia et al. 2000) 27 % of HBOC subjects *avoided* allogeneic RBC transfusion whereas all control subjects received at least one unit of allogeneic rbc's (randomization occurred at the occasion of the first transfusion trigger). In a trial of mixed surgical subjects (Sprung et al. 2002) there was a statistically non-significant reduction in RBC consumption. Biopure's extensively documented orthopedic surgical trial (Jahr et al. 2008) showed that 211 of the 350 HBOC 201 treated (60.3 %) subjects completely avoided pRBC, whereas none of those randomized to pRBC treatment did so. Treatment requirements varied widely in that 40.9 % and 21.6 % of subjects received only one unit of HBOC 201 and pRBC, respectively (this was a protocol mandated loading dose at entry to the trial, the first transfusion decision, a requirement for randomization), while 10.6 % and 2.7 % required >6 units.

Hemosol supported a Phase III trial in cardiac surgery (Hill et al. 2002; Greenburg et al. 2004). This multicenter trial was intended to demonstrate the utility of using Hemolink® to enhance intraoperative autologous blood donation (IAD), compared to conventional IAD whereby the volume deficit was made up by pentastarch. The report also included a matched set of contemporary historical controls from a single US academic hospital where transfusions in cardiac surgery were used liberally. Of those subjects who had their IAD augmented with HBOC 44 % did not require allogeneic rbc transfusion, compared to 24 % of those who did not have HBOC-augmented IAD. Moreover in the HBOC-augmented group there was a 53 % reduction in the total volume of allogeneic RBC transfusions, compared to the control group. Among the matched contemporary historical controls without IAD and with a liberal transfusion record 95 % received rbc transfusions of a total volume 9.8 times that in the HBOC-augmented IAD recipients. The frequency of non-RBC blood product transfusions were similar in

the two active study populations, but the total volume of blood product consumption in the HBOC-augmented arm was 47 % less than in the corresponding control arm. As the HBOC-augmented IAD subjects received their own *fresh autologous whole blood* collected intraoperatively, it is possible that this may have contributed to the reduced consumption of blood products in this group. There was no breakdown of the particular blood products used, and it is not possible to identify what particular allogeneic blood product(s) may have been spared by the HBOC-enhanced IAD. This trial clearly demonstrated the feasibility of HBOC-enhanced IAD, and the significant allogeneic RBC and blood product(s) savings it made possible.

The FDA-mandated endpoint was the clinically and statistically significant reduction of the proportion of subjects receiving allogeneic rbc transfusion. The explicit rationale of this end point was that allogeneic pRBC transfusions carry a risk of adverse "side effects", including the transmission of infectious agents, major and minor transfusion reactions, pulmonary complications (TRALI) and suppression of immune function that may compromise both resistance to infections and wound healing (see below). Since the 1980s the risk of transfusion transmitted infections, the major source of anxiety, has been drastically reduced and the frequency of the other adverse "side effects" attributable to transfusions are relatively small. It is also important to note that trauma *per se* is also accompanied by "side effects" similar to those attributable to pRBC transfusion; namely altered immune state. Avoidance of transfusions in the number of subjects, or reduction in the number of transfusions received by individual subjects, will only *reduce* the frequency of adverse outcomes associated with replacement of lost volume, but the relative magnitude of this reduction is difficult to estimate by separating those attributable to the circumstances from those attributable to blood transfusion. Hence, avoidance or reduction in allogeneic transfusion is a qualitative surrogate for another surrogate, namely the reduction of the *presumed* adverse outcomes associated with transfusion. In these circumstances, and based on the reported data, a *quantitative* balancing of risks and benefits becomes difficult.

The trials supported by Sangart used a different primary endpoint for efficacy: the reduction of frequency and/or duration of hypotensive episodes in hip surgery under spinal anesthesia (Olofson et al. 2011; van der Linden et al. 2011). The duration and frequency of hypotensive episodes in this setting was significantly reduced in those subjects treated with the Company's oxygenated polyethylene glycol-modified hemoglobin (MP4OX). This efficacy endpoint provides support for the notion that intraoperative hypotensive episodes can be better treated with this product than with a plasma expander (e.g. starch solution) and permits avoidance of vasopressor agents.

### *30.3.2 Safety Endpoints*

Safety endpoints generally involve the collection of all Adverse Events (AE's) and Serious Adverse Events (SAE's) with a preferably blinded assessment of the probable causal relationship of the event to the treatment. Because of the nature and spectrum of possible or probable adverse events an a priori power estimate needs to be made for the study to have sufficient predictive value. In various surgical settings this may or may not be feasible, because reliable, up-to-date data on the frequency of various adverse outcomes associated with various surgical procedures may or may not be available. In addition, studies may be required to be powered to account for the occurrence of rare (e.g. 2 in 1,000) but clinically highly significant events. None of the trials thus far reported would have the power to detect such an event.

In general, several of the studies reported that the aggregated Adverse Events were numerically more frequent among HBOC treated than in control arms of the studies. In the Baxter-supported emergency trials mortality and MODS scores were higher and the total number of SAE's was numerically but not statistically greater in DCLHb treated group. A number of reports contained tabulated data on the *group mean* of laboratory values at various time points. This form of reporting is of little value for comprehensive analysis, even if it shows numerically higher mean values for various analytes in the HBOC-treated arms, even when corrected for the hemoglobin-associated analytical artifact. One study stratified the frequency of AE's and SAE's by age group and found that age >70 years was a significantly higher risk factor for adverse outcomes in various categories (Freilich et al. 2009). In the cardiac surgery trial sponsored by Hemosol in which Hemolink® was used to enhance IAD Adverse Events were similar in the two treatment arms, and this was the only study that reported readmissions to hospital as a measure of the clinical significance of the adverse outcomes (Greenburg et al. 2004). In this study the incidence of readmission for the control patients was nearly twice that of the Hemolink® treated subjects (10 vs. 19). It is probable that RBC transfusion-mediated immune modulation and their sequelae may have been a contributing factor to the greater likelihood of readmissions.

Statistics applied to the number of AE's and SAE's do not offer clarity leading to clinically valid conclusions. Aggregated AE's may show a statistically significant difference without revealing clinical significance. Statistics applied to individual AE types are usually too few in number to achieve significance. The complexity and diversity of AE's and SAE's present particular difficulties in the interpretation of their clinical significance. By aggregating events their relationship to other events in the same subject are lost. It is for such reasons that it has been argued that the introduction of the particular clinical context would be relevant in the interpretation of safety data (Greenburg et al. 2008). In particular, the great variety and severity of underlying comorbidities in all populations studied may be an important confounding factor in assigning a singular causal relation to a treatment. When demographic and comorbidity data are aggregated or significant clinical details are

not recorded, very significant information may be lost. An example to illustrate this will be explored later in this chapter.

The safety data on HBOC products available to-date comprises a relatively small but important resource. It appears that with some variation the majority of adverse events reported are class effects, rather than individual and idiosyncratic effects specific to various products. Silverman and Weiskopf compared the reported adverse events in the HBOC treated and control arms of all human trials (Silverman and Weiskopf 2009). Admittedly, the aggregated data are not "clean" for a variety of known and unaccounted for reasons that also include varied definitions applied by the participating investigators for apparently similar clinical events. Moreover, it would appear that certain event types do not seem to occur with similar frequencies in product categories; rather, they seem to be "concentrated" with certain products. Is this a reflection of differences in the biological actions of different molecular species? Or is it a reflection of differences in the population profiles of different studies, in addition to differences in categorizing of events?

Table 2 in the review (Silverman and Weiskopf 2009) was compiled from data reported to the FDA by four companies (Baxter, BioPure, Hemosol and Northfield) comprising 2044 subjects exposed to various doses of HBOC in a variety of settings against 1772 control subjects. In the aggregate there were 30 % more deaths, and an almost doubling of serious cardiac events among HBOC-exposed subjects. "Pancreatitis" based on enzyme markers was reported more frequently in HBOC treated than in transfused control subjects in only two products. Other Serious Adverse Events were also reported with greater frequency in HBOC treated subjects. A separate group of subjects exposed in the BioPure orthopedic trial found that cardiac SAE's were more frequent in HBOC treated subjects and that AE's including elevations of troponin concentration were more frequent in the elderly (>70 years) stratum (Freilich et al. 2009), confirming that age is a recognized incremental risk factor for adverse outcomes in orthopedic surgery. If an imbalance in the age distribution in the two treatment arms was present (which is not necessarily evident in the reported mean or median values), an unaccounted bias may have been introduced. The case is similar for many of the comorbidities and other risk factors that are not accounted for in the safety analysis. These may affect single and aggregated outcomes.

Sangart Corp. has developed a PEG-modified hemoglobin product in oxygenated form (MP4OX, previously named HemoSpan®) and tested its efficacy to prevent perioperative hypotension in patients undergoing primary hip arthroplasty with spinal anesthesia (Olofson et al. 2011; van der Linden et al. 2011). These studies showed efficacy in reducing the number of hypotensive episodes relative to those treated with hydroxy-ethyl starch (66 % vs. 90 %), however, a greater number of AE's were seen in the MP4OX treated subjects, consisting mostly of laboratory abnormalities, troponin elevations and such symptoms as nausea. The clinical significance of elevations of troponin (not indicated whether of the cardiac (c) or skeletal muscle (m) isoform) cannot be assessed. Statistical significance was not found. An accompanying editorial expressed some doubts about the safety of using NO scavenging to treat hypotension and shock (Levy 2011).

The latest HBOC clinical trial to fail was The PHOENIX trial of PHP-hemoglobin tested for its efficacy in reversing distributive (septic) shock sponsored by Apex Bioscience and Curacyte Corp. The trial was terminated for futility on August 26, 2011 (http://www.curacyte.eu/news_11_08_26.htm Accessed on August 29, 2012). The trial enrolled a mixed population of subjects in septic shock of varying severity and failed because of higher mortality in the HBOC treated subjects. It is not clear whether or not shock severity was evenly distributed between the treatment arms.

Hemosol's Phase III trial in cardiac surgery (Greenburg et al. 2004) found a reduction of blood use and no differences in cardiac adverse outcomes. The frequency of adverse outcomes was similar between the treatment arms and mortality was low, but numerically greater in the control group. In a subsequent trial in cardiac surgery using larger volumes of Hemolink® the Company was required to pay particular attention to neurological outcomes, including mild cognitive impairment, against the background of the known incidence of "pump brain" in this setting. In the end, the study was halted not because of neurological complications but because of an excess of "cardiac events" diagnosed locally in HBOC treated subjects. In retrospect, a critical oversight was to allow the local investigators to diagnose cardiac events without any pre-specified unambiguous diagnostic criteria of biochemical marker elevations above the reference range and varied ECG and clinical findings. In this setting (see below) "cardiac events" of varying severity and significance are common. A central adjudication mechanism with pre-specified criteria used in all large scale cardiovascular drug trials would have been far more preferable. The Biopure orthopedic trial stands out among others for its use of an independent blinded event review by at least two non-treating physician reviewers of all case report forms. Following the termination of its trial, Hemosol conducted an intensive post hoc analysis of the data using stepwise logistic regression to exclude non-significant confounders and to identify significant contributing factors.[1] One of the significant differences between the HBOC and control groups was the prevalence of diabetes, a condition clearly identified as a critical incremental risk factor for adverse outcomes in this setting (Nalysnyk et al. 2003).

#### 30.3.2.1 Benefit Versus Risk, Certainty Versus Uncertainty

The true benefits of transfusion by pRBC depend on the magnitude of the deficit of oxygen transporting capacity and volume. They are unquestioned in the face of a critically low hemoglobin level (Pape et al. 2009). An attempt to estimate the potential benefits of transfusion *avoidance* by HBOC requires a quantitative examination of the risks of transfusions. A recent publication provides an

[1] The author was not provided access to the report on this exercise by TheraPure Corp the corporate successor of Hemosol Inc.

estimated incidence of adverse effects associated with allogeneic red cell transfusions (Tinmouth et al. 2006). Infection-transmission, a source of great anxiety in the past, has been drastically reduced to one in millions of transfusions, with the exception for the hepatitis B virus which is of the order of 1 in 30,000 to 80,000 transfusions. The frequency of bacterial contamination is of the order of one in 14,000 to 28,000 transfusions. Hemolytic transfusion reactions, a largely preventable event, occur in one in 9,000 transfusions. In addition, a number of studies link excess mortality and morbidity risk to transfusions (Meier et al. 2011). In a large cohort ($n = 27{,}789$) of transfused and un-transfused patients undergoing cardiac surgery (Rogers et al. 2009) the incidence of infections in patients receiving allogeneic transfusion was compared to those receiving autologous blood transfusion and those who did not receive a transfusion; the incidence of infection in recipients of allogeneic blood was nearly twice that in recipients of autologous blood and even lower in those who did not receive a transfusion (18.0 % vs. 9.7 % versus 6.6 %). A recent retrospective study from the Cleveland Clinic (Pattakos et al. 2012) is of substantial interest. This study compared outcomes in 322 Jehova's Witnesses undergoing CABG at the Clinic with a similar matched number of non-Witness patients selected by propensity analysis from 87,453 patients undergoing CABG from 1983 to 2010. Despite similar mean postoperative hematocrits in both matched groups, there were significant differences with respect to adverse outcomes between the transfused and untransfused populations: The untransfused Witnesses had significantly fewer perioperative myocardial infarctions, prolonged ventilation, reoperation for bleeding and shorter ICU- and hospital lengths of stay. Witnesses experienced numerically, but not significantly, fewer incidents of atrial fibrillation, renal failure requiring dialysis and septicemia. Estimated survival at 5 years was 86 % and 74 %, respectively, among the untransfused Witnesses and transfused patients, but the differences converged at 10 and 20 years. These findings seem to support the contention that transfusion *per se* is an independent risk factor for a variety of adverse outcomes in patients undergoing high-risk surgical procedures, possibly due to the accumulation of leukocyte-derived cytokines during storage (see below).

Although not a recognized risk of transfusions, the well-known storage lesion renders especially aged red cell units (>14 days) less than fully efficacious (Tinmouth et al. 2006; Bennett-Guerrero 2007; Kim-Shapiro et al. 2011) in immediately restoring oxygen transport capacity, and thereby preventing critical organ hypoxia (Grimshaw et al. 2011; Bonaventura 2007). Moreover, aged blood also contains leukocyte-derived cytokines that contribute to the development of a systemic inflammatory state (Bennett-Guerrero et al. 2007). The introduction of leukoreduction of red cell units ameliorates these risks and, as this procedure gains universal application, the risks will likely decline. It appears that reduction of the transfusion-associated risks is a worthwhile and ethically completely justifiable goal. However, when the comparison is made against HBOC replacement of allogeneic transfusion, the risks inherent in the HBOC need to be balanced against the risks of adverse outcomes associated with the pRBC transfusion.

The risk of transfusion-related adverse outcomes for a given subject is multiplied by the number of transfusions received, whereas there does not appear to be a clear relationship between number of HBOC units received and the frequency of adverse events. It is not clear how a *quantitative comparison* between transfusion-associated adverse outcomes and those apparently connected to HBOC replacement of pRBC is to be achieved. There is a need for the development of a robust method for a quantitative balancing of the clinically relevant risks and benefits of the avoidance of transfusion associated adverse outcomes, on the one hand, and the quantitative balancing of the *clinically relevant* (not necessarily statistically significant) benefits of the replacement of allogeneic transfusion by an oxygen carrier against the adverse outcomes associated with their administration, on the other.

The attribution of a causal relationship to a single factor or treatment (e.g. an HBOC) must be tempered by admitting of a number of uncertainties. As noted by Greenburg et al. (2008), the safety analysis employed in the completed studies are not sufficient to explore the numerous confounding factors that are at play because of the complex interplay of variations in medical-surgical practices, subject comorbidities, concomitant medications received, and many unknowns. Randomization and stratification of risk factors would improve a trial's validity.

An important source of confounding factors is the heterogeneity of clinical practice, most strikingly exemplified by the transfusion decision. In spite of protocol-mandated transfusion triggers the attending physician's clinical judgment, in the interest of patient safety, is paramount, and the "clinical judgment" was not necessarily based only on a priori defined objective criteria. A large cohort study illustrates this clearly in cardiac surgical practice (Rogers et al. 2009). The incidence of transfusion varies greatly (45–100 %) from hospital-to-hospital; about a third of the variability is attributable to between-hospital variations. One salient factor is evident in this survey: the probability of a patient receiving transfusion is highly dependent on the presence of some comorbid conditions: in the presence of diabetes, renal failure, myocardial infarction and congestive heart failure, chronic pulmonary disease and hypertension, transfusions were significantly more likely. In the HBOC trials imbalances in the prevalence of such confounding "demographic" factors between treatment arms and protocol deviations likely contribute to unintentional bias.

In contradistinction to the drawbacks of pRBC transfusion in the immediate period, HBOC's have been shown to be *effective* in contributing to oxygen transport, especially in those cases when erythrocytic hemoglobin is especially low (Gould et al. 1998). Moreover, when blood transfusion is not an option in life threatening anemia, when there are religious objections or in hemolytic anemias with high levels of antibodies, HBOC has been shown to be efficacious in maintaining life and enabling 23 of 55 subjects to survive with median hemoglobin at admission of 4.0 g/dL without transfusion (Mackenzie et al. 2010; Weiskopf 2010). When an HBOC is used to enhance IAD in cardiac surgery a clinically and statistically significant saving in blood and blood product use was demonstrated, and there was no difference in adverse outcomes, unlike in many of the other trials (Greenburg et al. 2004). The apparent benefit, without apparent excessive safety

risks demonstrated in one area suggests that in the practice of IAD HBOC's may find acceptance.

Any given outcome in a given subject is the result of many interacting factors of which the treatment is only one. The factors that may contribute to uncertainty in attributing causal relationship to outcome can be characterized as "known knowns", "known unknowns" and "unknown unknowables".

Among the "known knowns" are

- The natural history of the subject illness and its response to standard treatment;
- The likely rates of complications;
- Diversity of clinical standards of practices;
- Diversity of influence of e.g. age on outcomes.

Among the "known unknowns" are

- Comorbid conditions present in the population to be enrolled in the trial population;
- Existing coincident treatments received by the trial population;
- Diversity of conditions to be treated in the trial population (e.g. diversity of injuries and their extent).

Among the "unknown unknowables" are

- The response of individual subjects to the new treatment to be tested;
- Idiosyncratic response of any subject to the new treatment;
- Interaction between new treatment to be tested and any medications received by the trial subjects;
- Interaction of the diversity of standards of practice and new treatment to be tested;
- Any new event arising after randomization to treatment.

The impact of these contributing uncertainties can be mitigated by randomization, only if there is true randomization and stratification that homogenizes the diversities and balances the risk factors in the trial populations. Theoretically this can be achieved in one of two ways: either by restricting the subjects to be enrolled to a highly selected population excluding those who do not satisfy predetermined criteria of "homogeneity"; or alternatively by enrolling an unselected population of "all comers" without exclusions. The former allows a relatively smaller sample size for validity, but suffers from a lack of generalizability of the conclusions. The latter allows generalization of the conclusions, but at the expense of requiring a very large sample size. Between these two extremes in practice trials are designed to represent a compromise. However, major uncertainties may be unavoidable, as evidenced in the data of the Northfield trauma trial.

Data from this trial (Moore et al. 2009) illustrate the great difficulty of managing and executing a complex protocol in the major trauma emergency setting.

The primary efficacy outcome variable of the trial was statistical demonstration of "dual superiority-non-inferiority" of the initial HBOC resuscitation on 30-day

survival, compared to subjects treated according to standard of care, with transfusions as needed. Apart from the questions related to the appropriateness of initial resuscitation on 30-day survival, as noted above, a number of issues in the trial are worth exploring. A total of 714 subjects (the Modified Intent-to-Treat population, MITT) were enrolled in the trial; of these 350 and 364 were randomized to the HBOC and standard-of-care arms, respectively, thus constituting the *As-Treated* population (AT, $n = 349$ and 365). Subsequently, one subject was switched from the HBOC to the control arm. Because of a number of protocol violations involving the actual treatment received, this population was reduced to the *Per-Protocol* population (PP) of 279 HBOC-treated and 307 control subjects (total of 586 "appropriately randomized and treated"). The number of subjects thus removed was not balanced between the arms: 70 (20 %) from the HBOC arm and 42 (11.5 %) from the control arm.

The orthopedic surgical trial sponsored by BioPure (Jahr et al. 2008) also reported frequent protocol deviations (frequency 4.1 %) and discontinuations (frequency 5.7 %).[2]

The assumptions of the Bayesian theory (Jevning et al. 1994) can be illustrated as follows: An unknown number of identical marbles, except for being either black or white, is presented for sampling. Marbles are drawn one-by-one until a sample is collected whose number is determined by the power calculation that allows the inference on the total distribution of black and white marbles in the container with a given probability of being correct. The power calculation must be based on some informed opinion from past experience about the likely distribution of the two colors in the container. The theory does not allow for excluding from the sample marbles that are deemed to be of questionable property by post hoc *criteria,* particularly, when the number of exclusions is not balanced. This is the basis of the Intent-to-Treat analysis.

This is where the ideal theory collides with the reality of managing and executing a complex and highly demanding protocol in which a clinician's judgement of patient safety must trump protocol-mandated treatment in emergency settings. The reported number of protocol violations in the Northfield trauma trial was 128 (18 % of the 714 enrolled subjects), and of these 41 (32 %) of violations involved the wrong treatment, the numbers being approximately even (21 vs. 20) between the two treatment arms.

The primary efficacy endpoint was 30-day mortality. Table 30.1 summarizes mortality statistics as totals and percentages at 30 days in the populations analyzed.

Evidently a greater number and percentage of deaths were excluded and the relevant populations were reduced in the HBOC-treated than in the control arms. All this is not intended to impute any causative factor to the imbalances; it is merely used to demonstrate that in this setting, violations of the protocol may be

[2] The "deviations" should actually be characterized as protocol violations in those cases that involved failure to satisfy exclusion/inclusion criteria.

**Table 30.1**

| Population | HBOC-treated | Control |
|---|---|---|
| Modified intent-to-treat (MITT) | 47/350; 13.4 % | 35/364; 9.6 % |
| As treated (AT) | 46/349; 13 % | 36/365; 9.9 % |
| Per protocol (PP) | 31/279; 11.1 % | 28/307; 9.1 % |
| Number and % of deaths removed from AT | 15; 48 % | 8; 29 % |
| Number and % of subjects removed from AT | 70; 20 % | 58; 16 % |

unavoidable, and if they do occur, they may occur in an unbalanced manner that may affect the validity of inferences drawn from rigorous statistical procedures.

Recognizing the difficulty of cardiac diagnoses in the emergency trauma setting, the Northfield trauma trial (referenced above) subjected the investigator reported cardiac Adverse Events to an independent Cardiac Events Subcommittee adjudication blinded as to treatment, using a standardized decision algorithm. This review found a higher incidence of "probable infarction" than that reported by the investigators in the As-Treated population, and that the frequency of "probable infarction" was numerically greater in the PolyHeme® subjects, whereas the incidence of the other categories was similar between the treatment arms. Despite the rigorous review, the possibility of a relationship between cardiac events and PolyHeme® remained inconclusive.

The example of cardiac surgery illustrates other difficulties, especially in relation to the safety data.

Cardiac surgery is an important consumer of blood and blood products. Outcomes are greatly dependent on operative procedures. In the author's experience in the Hemosol cardiac surgery trial there were no a priori *defined uniform diagnostic criteria of a myocardial infarction* and, hence no uniformity in reporting cardiac AE's and SAE's. In this trial on-pump times ranged from 30 to >100 min. As this happens after randomization, departures from expectations may introduce unintentional bias. Prolonged on-pump time is likely to be associated with subsequent cardiac complications, elevated troponin levels, excessive bleeding and consequent requirement for larger replacement volumes and blood products. Likewise, the type of cardioplegia affects the likelihood of elevated troponin concentrations, the diagnostic significance of which in cardiac surgery is uncertain (Alpert et al. 2000). Recent guidelines established by the American College of Cardiology and European Society of Cardiology Working Groups have proposed that a perioperative diagnosis of post-CABG myocardial infarction (type 5) requires, among other criteria, a troponin concentration in excess of *five* times the 99th percentile of the reference range (Alpert et al. 2000; Daubert and Jeremias 2010), rather than just a value in excess of the local reference range. The most recent guidelines from the same Working Group recently released changed the troponin criterion for a CABG-related MI (type 5) to *ten* times the 99th percentile upper reference limit (http://www.medscape.com/viewarticle/769921?sssdmh=dm1.814252&src=nlconfnewst. Accessed Aug. 28, 2012). The use of anti-thrombolytics also has a major impact on blood loss and, hence, on transfusions. None of the cardiac surgery trials

specified a priori anti-thrombolytic use, since this would occur post-randomization. Their use is widely divergent between trial sites and operative teams. Such variations are not necessarily balanced evenly between treatment arms, especially if a large number of sites each contribute relatively small numbers of subjects. Such local variations in practice characteristics and unintentional imbalance in the clinical risk factors within the treatment arms may have significant impact on outcomes and on end points. Serious comorbidities are present in populations undergoing CABG, the most frequent being history of CV disease (46.7 %), diabetes (24.6 %), hypertension (52.1 %), according to a large scale systematic analysis based on 205,667 patients in 176 studies (Nalysnyk et al. 2003). These comorbidities have a very significant impact on the likelihood of adverse outcomes experienced. The analysis found that female gender (Odds Ratio, O.R. = 1.92), presence of diabetes (O.R. = 1.57), age > 70 years (O.R. = 2.42), prior heart surgery, prior MI and presence of hypertension significantly increased 30-day mortality and many of the non-fatal adverse outcomes (e.g. MI, stroke, GI bleeding and renal failure) (Nalysnyk et al. 2003). In addition, psychosocial factors (depression), independently of medical factors, also impact mortality and morbidity following heart surgery (Tully and Baker 2012). It is, therefore, clear that many factors may unbalance the treatment arms with respect to the probability of adverse outcomes and hence the trial's success in achieving its endpoint(s).

## 30.4 Conclusions

In the clinical trials of HBOC products various risk factors are present that may interact in complex and multiple levels. In a probabilistic sense it is not one single factor that accounts for the occurrence of most adverse outcomes but rather, the interaction of several self- and mutually-reinforcing clinical factors that account for most of the adverse outcomes. As proposed by this writer (Biro 2012) the effects of extracellular hemoglobin are most deleterious in the presence of *endothelial dysfunction* whereby the normal vasoregulation becomes tilted to predominant vasoconstriction because extracellular hemoglobin interferes with most of the nitric oxide-mediated regulatory signaling that is already compromised in endothelial dysfunction. Thus it could be expected that in the presence of *normal physiology*, HBOC would not present serious dysregulation, apart from an elevation of the systemic and pulmonary arterial blood pressures that can be counteracted by, e.g. breathing nitric oxide (Yu et al. 2008, 2009a, b). However, the majority of subjects participating in the HBOC clinical trials present with serious comorbidities that predispose to HBOC-mediated exacerbation of adverse outcomes. The imbalance in cardiac adverse outcomes in the Hemosol and other surgical trials may be accounted for by the interaction of coronary vascular endothelial dysfunction and HBOC.

It seems reasonable to conclude that in the presence of suitable available bank blood for transfusion the HBOC trials to date have not provided sufficient evidence

for a significant risk-reduction by substituting HBOC resuscitation for transfusion of pRBC. However, in a minority of cases HBOC may be especially efficacious when the risk of not using it exceeds the risks inherent in the HBOC safety profile. Such cases would be instances of critical anemia when bank blood is not an option for a variety of reasons. However, there is not sufficient evidence to assess the risk–benefit of the current HBOC's in specific circumstances when blood is not available in the field, to provide additional oxygen carrying capacity to enable survival to delayed definitive treatment. This may include some military applications or disaster scenarios. One study (Greenburg et al. 2004) has shown evidence that using an HBOC to enhance IAD, at least in cardiac surgery, is a useful adjunct in that it provides autologous fresh whole blood for transfusion in the intra- and postoperative period, saves allogeneic blood and blood products, and is seemingly without additional safety hazards. An estimate showed that an average one-third reduction in allogeneic rbc transfusions in cardiac surgery would have a significant impact on total blood consumption. The IAD procedure, unlike scheduled preoperative autologous blood donation, requires no specific infrastructure and provides immediately available fresh whole autologous blood for transfusion. The fact that blood collection is a continuously ongoing effort without the possibility of creating stockpiles for emergencies indicates that there is an unmet need. Hemoglobin, appropriately modified, would still be the suitable candidate to fill several areas of the unmet need.

Safety data as currently reported are not sufficiently explicit to base conclusions on the balance of benefit and risks in the area of HBOC's. Efforts to quantify better their clinical significance will be required. Such quantitative measures as the rates of readmission, prolonged ICU and hospital lengths of stay, etc. will be welcome additional measures to help consideration of clinical risk–benefit assessment.

In some trials a noticeable reduction in the consumption of *blood products* occurred in HBOC treated groups (Sloan et al. 1999a; Greenburg 2004). The explanation of this phenomenon is elusive, but should be looked at. A robust model for the assessment of clinically relevant risk benefit comparison is needed.

It would appear to this writer that to an important extent the clinical development of HBOC's has been affected by corporate priorities. In the case of single-vehicle "start-up" entities fundraising and the urgency expressed by shareholders and analysts expecting rapid and unrealistic profit targets have adversely affected the course of development. At the time when "irreversible" development decisions were made the preclinical data obtained on normal animals were encouraging and minimal information was sought in disease models exhibiting endothelial dysfunction which was just beginning to be recognized. Newly developed products need to be tested in animal models representing the conditions that the products will encounter in clinical use, as recommended by the NIH/FDA/DOD Interagency Working Group on Oxygen Therapeutics (Boston, July 26, 2011) (http://www.nhlbi.nih.gov/meetings/workshops/therapeutics.htm. Accessed Aug. 29, 2012).

## 30.5 Recommendations

Several important lessons arise from the completed HBOC clinical trials.

Balancing efficacy endpoints against safety data need to be more explicit and quantitative. A robust model for quantitative and objective comparison of clinically relevant benefit and risks of adverse outcomes would offer increased confidence and validity to trial findings.

Trauma trials in the emergency setting are extremely difficult and fraught with organizational complexities and the potential of substantive protocol violations that may significantly impact outcomes. It is important to specify a priori the definitions of significant outcomes and events and to establish post hoc blinded and centralized adjudication of events by criteria established a priori. In particular, cardiac adverse events should receive a centralized adjudication process along the lines used in major cardiovascular drug trials (TIMI, Thrombolysis In Myocardial Infarctions and GRACE, Global Registry of Coronary Events) (Eagle et al. 2004; Anderson et al. 2007). The use of unambiguous standardized nomenclature of clinical events is essential. Disparities in clinical practice between clinical sites should be minimized, perhaps by stratification. The statistical analysis plan should include analysis by step-wise logistic regression to exclude non-significant confounders and to identify significant risk factors that result in unbalanced treatment arms.

Trials in elective surgery also need to have a priori standardized definitions of safety events and careful statistical analysis of confounders and identification of imbalances in treatment arms. An attempt should be made to establish some sort of hierarchy and equivalence between disparate adverse events. It is probably not sufficient to count *adverse events, severe adverse events and serious adverse events* only. For example, is there a similarity in clinical impact between a transfusion reaction and pancreatitis of varying severity?

Newly developed products need to be tested in animal models representing the conditions that the products will encounter in clinical use, as recommended by the NIH/FDA/DOD Interagency Working Group on Oxygen Therapeutics (Boston, July 26, 2011) (http://www.nhlbi.nih.gov/meetings/workshops/therapeutics.htm) Accessed Aug 29, 2012).

## 30.6 A Postscript on Mechanisms

A mechanism has been proposed to account for some of the observed adverse outcomes that may be attributable to a synergism between extracellular hemoglobin and endothelial dysfunction (Biro 2012). Endothelial dysfunction is not a monolithic binary entity; rather, it is highly variable in its severity and location among organs in any individual and between individuals. It is present in a number of highly prevalent diseases, in the aged and is likely present in a large segment of

the population undergoing elective surgery. Extracellular hemoglobin exacerbates the deleterious effects of endothelial dysfunction. The synergistic actions occur at a number of levels, by interference with nitric oxide-mediated mechanisms including a shift to vasoconstriction and oxidative stress. It is thus conceivable that endothelial dysfunction, likely present to varying degrees in the trial population and, if present in the coronary circulation, would likely contribute to adverse cardiac outcomes by inappropriate vasoconstriction at sites of atherosclerotic obstructions. It is likely that such HBOC synergistic actions are not specific to any given product characteristic. However, because of the diversity of contributing factors, the relative contribution of interactions is difficult to ascertain. Hence, undetected dissimilarities in treatment arms may become critical confounders. Only careful analysis of all comorbidities and other risk factors could identify risk factor imbalances, if any.

In addition to its actions of extracellular hemoglobin, nitric oxide modulation is an important factor in matching perfusion and mechanical activity following experimental myocardial ischemia (Kingma et al. 2011), and is also an important signaling molecule *within* the cardiomyocyte mediating the actions of the GLP-1 receptor and the phosphokinase c pathway (Hausenloy and Yellon 2012), as well as the ryanodine receptor and calcium handling, with important downstream effects upon mitochondrial and contractile function (Bonaventura and Gow 2004). It is an intriguing but open question whether extracellular HGb can modulate intracardiomyocytic NO-dependent regulatory mechanisms, and thereby HGb based scavenging can contribute to adverse cardiac outcomes.

The author has no interest in any commercial developer, manufacturer or clinical site involved in HBOC or related product.

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# Chapter 31
# Compassionate Use Cases Treated with Hemoglobin-Based Oxygen Carriers

**Paula Moon-Massat and Daniel Freilich**

*"I had one litre of blood left in my body," Ms Coakley said, a 33-yr-old who was deliberately placed in a coma during the crucial medical procedure in which she received 10 units of a haemoglobin-based oxygen carrier after almost bleeding to death from an automobile injury. "They did everything they could, I am so grateful." (www.news.com.au, 2011)*

## 31.1 Introduction

One of the most elusive goals in the modern medical era has been to design a safe and effective asanquinous oxygen therapeutic (OT). After 80 years of research and dozens of clinical trials, most of the 17 billion people in the world are still without an alternative to blood; South Africa and Russia are currently the rare exceptions (Amberson, 1933; www.opkbiotech.com, 2012).

While it is difficult to estimate the number of people who are unable to receive a blood transfusion for medical (i.e., auto-immune mediated hemolytic anemia [AIHA]) or logistical (i.e., far-forward combat casualties or rural civilian settings) reasons, it may be those who refuse blood for religious reasons who are the largest potential population to benefit from an approved OT. The most recognized of these religious groups are the Jehovah Witness's and, in the United States alone, their

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population exceeds 1.20 million while, worldwide, there are over 7.66 million members and 109,400 congregations (Watchtower Bible and Tract Society 2012). A retrospective observational study compared Jehovah's Witness patients with severe symptomatic anemia from any cause and who refused blood to similarly matched patients treated with allogeneic red blood cell (RBC) transfusions (Beliaev 2012). These blood transfusions reduced the mortality and shock states in these patients (Beliaev 2012). It is precisely in this population, more than any other, where a patient's willingness to receive an experimental fluid in a "compassionate use" situation, outside the realm of a controlled clinical trial, has set the stage for continued research in OTs.

This chapter reviews the literature of nearly 70 case reports in which an OT was used in an emergency, life-threatening situation when a blood transfusion was not an option. Most of the patients were Jehovah Witnesses but other indications for treatment show the diversity of potential uses of an OT. All but one citation report the results of a single individual patient and describe the effects of receiving an HBOC as the OT. The details and importance of these cases, individually and collectively, will be discussed.

## 31.2 Compassionate Use Regulations

"Compassionate use" (CU) is a phrase that describes the "expanded access" of an investigational, unapproved drug outside of a clinical trial to treat an individual patient (or intermediate-size group of patients with similar treatment needs) that has a serious or immediately life-threatening disease and for whom there are no satisfactory alternative treatment options. There are no Food and Drug Administration (FDA) regulations or policies specifically defining the term "compassionate use". Instead, the FDA refers to a CU request as a "single patient Investigational New Drug (IND)" study. Since 1987, the FDA has had rules that permit patients, under specific circumstances, to access drugs still in development and these rules were amended in 2009 to ensure "broad and equitable access to investigational drugs for treatment" (www.FDA.gov 2012). Outside the U.S., programs that enable access to drugs in the pre-market phase are referred to by a variety of names including "named patient programs," "named patient supply" and "temporary authorization for use" (Helene 2010). In the European Union, named patient programs also allow patients to access drugs in the time period between centralized European Medicines Agency (EMEA) approval and launch in their home countries (Ericson 2005). The FDA regulations, and these other programs that follow similar criteria, require the following general conditions before granting expanded access (Federal Registrar, 21 CFR Parts 312 and 316, 2009):

1. The patient must have a serious condition or disease for which there is no comparable alternative therapy available.
2. The patient must be unable to participate in a clinical trial.

3. The potential benefit must outweigh the potential risk of using the treatment.
4. There should be no impact on the completion of a clinical trial or the drug's approval.

There is a progression of individual stages that must be completed before a drug is made available to a patient under an expanded use protocol. The attending physician takes the *initial step* in the process by making the critical decision that a particular patient may be appropriate for and benefit from a particular, unapproved drug. The physician must provide documentation that all of the criteria are met for the FDA to authorize expanded access use, including informed patient consent. Not all physicians are aware that such a drug may be available for use in a particular medical condition and not all physicians are able or willing to manage the additional work involved when using an investigational drug for patients in their care.

The *second step* in the CU process is for the pharmaceutical company to agree to provide the investigational drug to the physician. Often, the public is not aware that a company cannot be forced to provide a potentially "life-saving" drug. This decision lies entirely with the pharmaceutical manufacturer and there are multiple factors a company must consider before supporting a CU case. For example, supply of the drug may be limited, leading to competition for the drug between designed clinical trials and CU cases. Unlike data obtained from a clinical trial, CU cases do not provide information that will necessarily advance the clinical development and regulatory approval of the drug. In fact, by its very nature, the CU of a drug is in a less controlled setting and in patients with very advanced or life-threatening diseases which could, in turn, "lead to adverse reactions that might raise spurious safety concerns about the drug" (Temple 2001). Thus not all OT companies have the resources or the inclination to embark on a CU program.

The *third step*, once the physician identifies a need for the drug and the manufacturer indicates a willingness to provide the drug, is for the FDA, or equivalent agency, to grant approval to use the drug in that particular situation. In the typical CU single patient IND, the patient's physician generally has limited information about the drug being requested and it is the FDA's responsibility to provide an objective decision on the risk:benefit ratio to the individual who would be receiving the drug. This independent scientific consideration by the FDA is a critical and essential component of ensuring patient protection, when one is considering drugs about which a variable amount is known and where potential toxicity may occur. The FDA is permitted to approve individual expanded access cases by telephone, and, as provided by the regulation covering Institutional Review Boards (IRBs) and informed consent, if there is insufficient time for an IRB to meet, a drug may be administered without IRB approval *a priori*. The proper supporting documentation must be submitted within 15 days to the FDA and notification of the IRB approval is required within 5 days to the FDA (Federal Registrar, 2009 and FDA, 21CFR 56.104, 2002).

Given the number of stages, people, and agencies involved in obtaining expanded use approval, the time between the physician's decision to try a drug and the patient actually receiving the drug can be surprisingly expedient. Nonetheless, it is absolutely critical that this time delay be shortened by anyone who is attempting to obtain an OT drug for CU. It appears that the chances of survival can be substantially increased by minimizing the time between the request for and administration of the OT (MacKenzie 2010).

## 31.3 Overview of Oxygen Therapeutic Compassionate Use Cases

The first OT to be given under a CU request was a first generation OT, Fluosol-DA (Alpha Therapeutics, Los Angeles, California) which contained an emulsion of two perfluorochemicals, perfluorodecalin and perfluorotripropylamine. Approved for use during percutaneous transluminal coronary angioplasty in 1989 (Wikipedia 2012), Fluosol-DA was used off-label to treat a 57-yr-old male Jehovah Witness 6 years before the next report of an OT being administered in a CU case. This patient had lost 7 units of blood intraoperatively during a radical prostatectomy for prostate cancer (Marelli 1994). On post-operative Day 2, his hemoglobin (Hb) concentration had decreased to 30 g/L, he was showing symptoms of anemia (tachycardia, tachypnea, lethargy, and diaphoresis) and he then received 5 doses of Fluosol-DA over 5 days while breathing high inspired oxygen concentrations. The patients' mixed venous oxygen saturation ($SvO_2$) rose from pre-treatment values of 50—55 % to post-OT treatment values of 70—80 %. The patient, who was re-dosed with Fluosol-DA whenever the $SvO_2$ fell below 60 %, eventually recovered from his anemia and was successfully discharged from the hospital. This historical case paved the way for conceding that OTs may have a role in emergency medicine; temporarily alleviating oxygen deficits, and increasing the chances of patient survival.

All 68 of the subsequently reported CU OT treatments (in 67 patients) were with an HBOC (Table 31.1): 60 were administered HBOC-201 (Biopure Corporation, Cambridge, MA), 5 were administered Polyheme (Northfield Laboratories, Evanston, IL) and 3 were administered Hemolink (Hemosol, Toronto, Ontario, Canada). It should be noted that prior to the 54 HBOC-201 CU cases reviewed by MacKenzie et al. (MacKenzie 2010), only four HBOC-201 cases were previously published. With so many additional unpublished HBOC-201 CU cases, and given that none of these companies are currently in existence (Biopure is now OPK Biotech), this suggests that the cited number may be an under-representation of the true number of patients to receive OT in an emergency CU situation.

**Table 31.1** A summary of the literature on CU patients who received an HBOC for anemia or blood

| HBOC Dose/ Duration | Nadir Hb (g/dL)/ Hct (%) | Plasma Hb (g/dL) | Outcome/Treatment comments | Case description/Significance | Reference |
|---|---|---|---|---|---|
| *HBOC-201* (30 *g*/250 *ml Unit*) | | | | | |
| 11 U (330 g)/7 d | 4.4/1.5 | 3.4 | Survived/No hypertension or AEs reported; resolution of ECG ischemic changes | 21-yr-old F. AIHA; 1st received RBC without improvement. Co: septic shock, hypotension requiring vasopressor support, lactic acidosis, osteomyelitis/ 1st CU HBOC case | 2000, Mullon* |
| 7 U (210 g)/4 d | 3.5/ND | 1.7 | Survived acute episode; Treated on Days 5−7 and died Day 37 from abdominal sepsis./No hypertension or AEs reported | 50-yr-old M JW; Anemia after GI hemorrhage. Co: 3 yr post-renal transplant, hepatitis C, hypercholesterol, diabetes mellitus, acute respiratory failure, hypotension/Severe, life-threatening anemia can be treated with concurrent HBOCs and rHuEpo, without the transfusion of allogeneic blood | 2002, Gannon* |
| 41 U (1230 g)/18 d | 1.9/10 | 3.6 | Survived acute episode; Died Day 18/ Possible cytokine release; PET scan showed homogenous brain oxygenation during treatment | 52-yr-old F JW; Anemia after myelosuppressive chemotherapy. Co: leucopenia, thrombocytopenia, pulmonary edema, cardiac hypokinesis, renal failure/Long-term use of high dose HBOC-201 without additional RBC support | 2005, Agrawal* |
| 1 U (30 g)/6 h | 2.0/ND | ND | Survived/No hypertension or AEs reported. Resolved tachycardia, hypotension and began feeding | 23-mo-old F JW; Severe sickle cell anemia; Co: heart failure, hypotension; required vasopressor support/Youngest pediatric (toddler) case | 2007, Stefan* |

(continued)

**Table 31.1** (continued)

| HBOC Dose/ Duration | Nadir Hb (g/dL)/ Hct (%) | Plasma Hb (g/dL) | Outcome/Treatment comments | Case description/Significance | Reference |
|---|---|---|---|---|---|
| 6 U (180 g)/2 d | 4.5/11.6 | 1.4 | Survived/No hypertension or AEs reported | 23-yr-old M JW; hit by motor vehicle (multiple rib fractures, iliac wing and acetabular and symphysis fractures, pneumothorax, grade 3 liver laceration, hepatic and iliac artery tears); Co: lactic acidosis/1st blunt trauma CU case | 2008, MacKenzie |
| 2 U (60 g)/1 d | 1.7/6.2 | ND | Survived/metHb (7.9 %) only AE | 28-yr-old M. Hb H disease with Evan's syndrome (AIHA and ITP). Co: sustained reticulocytopenia (0.1 %), lactic acidosis, respiratory distress, vasopressor support for hypotension/1st use of HBOC as adjunct to conventional RBCs while awaiting response to conventional treatment in patient with combined AIHA and ITP | 2008, Pachinburavan |
| 8 U (240 g)/3 d | 3.2/ND | 3.8 | Died (life support withdrawn on Day 10)/ No AEs or hypertension associated with HBOC but interpretation difficult; marked increase in % brain $O_2$ sat, central venous $O_2$ sat, and hemodynamics. Author comment that death may have been from massive reperfusion injury due to delayed repayment of severe cerebral $O_2$ debt | 21-yr-old M JW. Hit and dragged by motor vehicle (brain CT revealed extensive subdural, intraparenchymal, and subarachnoid hemorrhage, and brain AIS score = 4). Co: coagulopathic, and acidotic/1$^{st}$ HBOC case with severe TBI and massive hemorrhage | 2009, Marinaro |
| 15 U (450 g)/12 d | 3.6/ND | ND | Survived/metHb (13.6 %) only AE | 36-yr-old F JW; acute lymphoblastic leukemia (ALL) | 2010, Donahue |

(continued)

**Table 31.1** (continued)

| HBOC Dose/ Duration | Nadir Hb (g/dL)/ Hct (%) | Plasma Hb (g/dL) | Outcome/Treatment comments | Case description/Significance | Reference |
|---|---|---|---|---|---|
| Median 8 U (240 g)/4 d | Median 3.9/ ND | ND | 42 % survived/The 3 most reported AEs were increase in blood pressure (systolic blood pressure > 160 mm Hg), an increase in liver enzymes (outside normal range), and metHb (outside normal range). See Table 31.2 and 31.3 for case summaries | 55 Treatments in 54 patients. See Table 31.2 and 3 for case summaries/ Only report of CU case series | 2010, MacKenzie |
| 5 U (150 g)/2 d | 2.9/ND | ND | Survived/No AE noted; cardiac hypoxia (troponin elevation, ECG changes) resolved | 32-yr-old F JW; motor vehicle accident (Imaging showed a fractures of right orbit and maxilla, bilateral ribs, left distal humerus, a comminuted open left femoral shaft, an unstable T12/L1 dislocation (60 % off-ended), and multilevel spinous process and transverse process fractures and and grade 4 splenic laceration, jejunal injury)/1st case describing reversal of documented cardiac hypoxia secondary to anemia following trauma | 2011, Fitzgerald |
| **Polyheme (50 g/500 ml)** | | | | | |
| 5 U (250 g)/ND | 3.2/ND | 6 | Survived/No treatment comments provided | 44-yr-old F JW; motor vehicle accident (temporal subarachnoid hemorrhage; orbital tripod fracture; facial lacerations, bilateral pulmonary contusions; three rib fractures; and grade 1 splenic laceration)./1$^{st}$ Polyheme CU case | 2002, Cothren |

(continued)

**Table 31.1** (continued)

| HBOC Dose/ Duration | Nadir Hb (g/dL)/ Hct (%) | Plasma Hb (g/dL) | Outcome/Treatment comments | Case description/Significance | Reference |
|---|---|---|---|---|---|
| 12 U (600 g)/∼ 13 d | 4.1/11.9 | ND | Survived/No hypertension or AEs reported. Decrease in pre- to post-infusion heart rate from 98 to 80 bpm ($p = 0.014$) | 26-yr-old F JW; Presented with vasoocclusive crisis from sickle cell disease and developed acute chest syndrome (ACS; bilateral pulmonary infiltrates, pulmonary embolism), staphylococcal bacteremia, profound anemia and thrombocytopenia/1st case of sickle cell patient with ACS | 2002, Lanzkron |
| 2 U (100 g)/1 d | 2.9/ND | ND | Survived/No hypertension or AEs reported | 51-yr-old F JW; Persistent colonic bleeding | 2004, Allison |
| 18 U (900 g)/16 d | 2.9/ND | ND | Survived/Resolution of dyspnea, tachycardia, lactic acidosis and ischemic ECG signs | 39-yr-old F JW; abruptio placentae with fetal death at 31 weeks gestation complicated by maternal DIC/Highest Polyheme dose over longest duration | 2004, Cothren |
| 4 U (200 g)/5 d | 2.7/ND | ND | Survived/No hypertension or AEs reported. Resolution of tachycardia, severe fatigue, dyspnea, headaches, blurred vision | 55-yr-old F JW; history of B-cell lymphoma; submyeloablative stem cell transplant complicated by delayed graft failure and subsequent anemia. No co-morbidities./1st case with graft dysfunction after stem cell transplantation | 2006, Smith |
| **Hemoglobin raffimer (25 g/250 ml)** | | | | | |
| 20 U (500 g)/6 d | 3.2/ND | ND | Survived/Scleral icterus and blood pressure increase (to 180/54 mm Hg), 7.9 % metHb and 3.8 % COHb with reduced $\%O_2$ sat. Transient asymptomatic increase in bilirubin, amylase and lipase. CK-MB normal on Day 1 and 5. Resolution of tachycardia, ST-segment depression, mental status changes, and hypotension | 53-yr-old F JW; total hip replacement developing acute chest syndrome./1st Hemoglobin raffimer CU case | 2005, Landzinger |

(continued)

**Table 31.1** (continued)

| HBOC Dose/ Duration | Nadir Hb (g/dL)/ Hct (%) | Plasma Hb (g/dL) | Outcome/Treatment comments | Case description/Significance | Reference |
|---|---|---|---|---|---|
| 5 U (125 g)/6 d | 3.1/9.3 | 1.5 | Survived/Transient pulmonary hypertension, volume overload, fever developed. However, HBOC improved $DO_2$ and permitted surgery | 20-yr-old F JW; Severe anemia due to menorrhagia that required uterine dilatation and curettage. Co: none./ HBOC served as adjunct to therapy, allowing successful surgery | 2004, Shander |
| 2 U (50 g)/6 h | 2.6/7.9 | 0.7 | Survived/Mild abdominal pain, transient elevation in blood pressure, transaminases and amylase. Tachypnea and tachycardia resolved | 14 yr old F JW with ITP./1st use of HBOC in pediatric (adolescent) patient | 2002, Anton |

AEs = Adverse events; AIHA = Autoimmune hemolytic anemia; Co = Comorbidities; CT = Computed tomography; DIC = Disseminated intravascular coagulation; F = Female; ITP = Immune thrombocytopenia; JW = Jehovah's Witness; M = Male; N/A = Not applicable; ND = No data; U = Units
*included in MacKenzie, 2010

**Table 31.2** Survival outcome of HBOC-201 treatment (N = 55) in CU cases by age, gender, co-morbidities, reason for enrollment and cause of anemia

| | Overall | Survivors | Non-survivors | P |
|---|---|---|---|---|
| *Number of patients* (%) | 55 | 23 (42 %) | 32 (58 %) | |
| *Age* (y) | 50 (26) | 43 (27) | 52 (24) | 0.059 |
| *Years, N* (%) | | | | |
| <60 | 42 (76) | 19 (45) | 23 (55) | |
| ≥60 and < 75 | 10 (18) | 3 (30) | 7 (70) | |
| ≥75 | 3 (5) | 1 (33) | 2 (67) | |
| *Gender* | | | | 0.220 |
| Female | 33 (60 %) | 16 (65 %) | 17 (53 %) | |
| Male | 23 (40 %) | 7 (35 %) | 15 (47 %) | |
| *Co-morbidity* | | | | |
| Cancer | 11 | 0 | 11 | **0.001** |
| Cardiovascular disease[a] | 24 | 12 | 12 | 0.279 |
| Diabetes | 7 | 3 | 4 | 1.000 |
| Renal disease[b] | 17 | 6 | 11 | 0.512 |
| Other comorbidities | 17 | 9 | 8 | 0.263 |
| Comorbidities per patient (median ± interquartile range) | 1 (1) | 1 (2) | 1 (2) | 0.993 |
| *Reason for enrollment* | | | | 0.143 |
| Transfusion refused | 46 (84 %) | 17 (74 %) | 29 (90 %) | |
| Medical[c] | 9 (16 %) | 6 (26 %) | 3 (10 %) | |
| *Cause for anemia*, N (%) | | | | |
| Medical | 19 (35) | 8 (15) | 11 (20) | 0.975 |
| OBGYN | 6 (11) | 4 (7) | 2 (4) | 0.223 |
| Surgical | 25 (45) | 9 (16) | 16 (29) | 0.425 |
| Trauma | 5 (9) | 2 (4) | 3 (5) | 1.000 |

Data are presented as count (%) or median (interquartile range). Reprinted from MacKenzie (2010) with permission by Wolters Kluwer Health, publishers

[a] Including hypertension; [b] Including patients with acute renal failure, end stage renal disease, nephropathy and chronic renal failure following renal transplant; [c] Alloimmunized or hemolytic reaction; GI, gastrointestinal; OBGYN, obstetrical-gynecological

## 31.4 Limitation and Contribution of Case Reports

Case reports are considered as no more than anecdotal evidence with a number of important limitations. The efficacy and safety data in a case report is limited by the absence of any type of randomly allocated control group. There is no standard treatment protocol thus resulting in non-uniformity of dosage and timing of administration. There is variable and sometimes even an absence of data and no system to verify the data that was collected (Vandenbrouke 2001). Thus, firm conclusions about efficacy or safety cannot be drawn from case reports. On the other hand, these case reports allow communications to occur in the medical community about OTs being used in situations that are outside the realm of a

clinical trial. They showcase unique applications for treatment and unexpected results as well as permit a better understanding of the underlying mechanisms of this class of drugs. Perhaps most importantly, they can reveal potential trends or correlations that can lead to hypothesis generation, particularly when they can be analyzed in large enough numbers. These data-driven hypotheses can guide the design of future pre-clinical studies and/or clinical trials. Thus despite lacking the scientific strength of a controlled clinical trial, these CU cases have an important role in advancing the medical science of OT.

## 31.5 Oxygen Therapeutic Compassionate Use Case Series

A particularly vital contribution to the OT literature was the series of 54 CU patients who received HBOC-201 (MacKenzie 2010). This report provided useful information on administering an OT to severely anemic subjects (median Hb of 40 g/L) during medical, surgical, obstetrical and traumatic emergencies and provided insight into possible reasons for success or failure when using an OT. Their retrospective analysis of widely differing scenarios provided a unique opportunity to evaluate the impact of the duration of time, from the development of anemia (defined as a Hb $< 80$ g/L) to the administration of the first HBOC treatment, on patient survival. When these HBOC-201 cases were evaluated collectively (Tables 31.2 and 31.3), the difference between a delay of 3.2 days or a 4.4 days (from the time of anemia to administering the OT) was, literally, the difference between life and death ($p = 0.027$). Interestingly, the Hb concentration, *by itself*, at time of IND ($p = 0.730$), on admission to the hospital ($p = 0.948$), or immediately before HBOC treatment ($p = 0.120$) was not significantly different between survivors and non-survivors. However, the impact of combining *duration* and *severity* of anemia (termed "Hb deficit—duration product") was significantly different ($p = 0.039$) between survivors and non-survivors. On a case-by-case basis, the effect on survival of either the duration of the anemia or the Hb deficit—duration product would not have been possible and, to date, there is not a single HBOC clinical trial where the timing of the HBOC showed a difference in survival outcome.

The subsequently reported 14 additional OT CU cases, using any of the available OT, showed a similar pattern of data and support the study's conclusions. The survivors were often younger patients who received the OT within a shorter period of time following the onset of their anemia. In fact, if data from these additional cases are included with the MacKenzie series and using a two-sided *t* test to compare age between survivors and non-survivors, then patient age (which was not quite statistically significant [$p = 0.059$] in the MacKenzie series) becomes statistically significant ($p = 0.0001$) with younger patients (36.0 y, 14–75 y; median, interquartile range) more likely to survive than older patients (52.0 y, 19–78 y).

**Table 31.3** Survival outcome in CU cases by severity of pre-treatment hemoglobin concentration, duration from known anemia (≤ 80 g/L) to HBOC-201 treatment, hospital durations and units of HBOC-201 administered (N = 55 HBOC-201 treatments)

| | *Overall* ( *n*= **55**) | *Survivors* ( *N*= **23**) | *Non-survivors* ( *N*= **32**) | *P* |
|---|---|---|---|---|
| Hemoglobin [Hb][a] (g/L) | | | | |
| [Hb] on admission | 79 (81) | 84 (77) | 77 (83) | 0.948 |
| [Hb] at time of IND request | 40 (21) | 45 (22) | 39 (17) | 0.073 |
| [Hb] before first HBOC-201 treatment | 39 (20) | 45 (21) | 38 (14) | 0.120 |
| Change in [Hb] from admission to prior to first HBOC-201 treatment | -37.5 (75.5) | -42.5 (74) | -36.5 (79) | 0.657 |
| Duration (days) | | | | |
| Admission to resolution | 14 (15) | 15.5 (13) | 13 (18) | 0.398 |
| Admission to first HBOC-201 treatment | 5 (9) | 3 (6) | 7.5 (10) | 0.072 |
| IND request to first HBOC-201 treatment | 1 (2) | 1 (2) | 1 (2) | 0.817 |
| Anemia (≤ 80 g/L) to first HBOC-201 treatment | 4.0 (6) | 3.2 (4) | 4.4 (8) | **0.027** |
| HBOC-201 treatment | 4 (6) | 5 (7) | 3 (6) | 0.612 |
| First HBOC-201 treatment to resolution | 7 (13) | 12 (10) | 4.3 (8) | **0.005** |
| Hb-deficit—duration product (g/L x days) | 190 (220) | 162 (130) | 211 (298) | **0.039** |
| HBOC-201 Treatment (U) | 8 (8) | 7 (9) | 8 (7.8) | 0.549 |
| Total units | | | | |
| ≤ 4 | 18 | 8 | 10 | |
| <4−6 | 6 | 3 | 3 | |
| <6−10 | 14 | 5 | 9 | |
| <10 | 17 | 7 | 10 | |
| Loading dose | 2 (0) | 2 (0) | 2 (1) | 0.109 |

Data are presented as median (interquartile range). Reprinted from MacKenzie (2010) with permission by Wolters Kluwer Health, publishers

IND = Investigational new drug; [Hb] = Hemoglobin concentration; HBOC = Hemoglobin-based oxygen carrier

## 31.6 Did the Patient "Need" a Transfusion?

The classic critique against the usefulness of the OT in these cases is that "*the literature is replete with cases of patients who were Jehovah's Witnesses who refused transfusions and survived despite hemoglobin concentrations of less than 5 g per deciliter. Hemoglobin-based oxygen carriers are an interesting concept, but so far, there are few data … supporting their use in humans.*" (Hardy, 2002)

Ironically, the same assessment could be made about the efficacy of blood transfusions. It is pointless to dispute the specific level of Hb that triggers an individual physician's decision to use an OT, especially when there is no consensus on the appropriate Hb trigger to transfuse blood (Shander, 2011; Carson, 2011; Carson 2002; Consensus Conference 1988). There are many other factors

involved, besides the Hb concentration, which will increase the likelihood of an ischemic adverse event including whether or not the patient has an underlying cardiac disease, neurological disorder, or renal disease, or if the patient is elderly, critically ill, or undergoing an orthopedic surgery (MacKenzie 2010). Therefore a careful review of the co-morbidities and the entire clinical scenario is part of the challenge in showing efficacy with a modality such as an OT. The Lanzkron case report describing the use of the OT Polyheme is an example of the complexity of these cases and the difficulty in determining the efficacy of the product. In that report, Polyheme appeared able to support a markedly anemic (50 g/dL Hb) patient having multiple complications that included sickle cell disease, acute chest syndrome, pulmonary embolism, staphylococcal bacteremia and impending respiratory failure and who also refused RBC transfusions (Lanzkron 2002). As indicated in the MacKenzie series, the Hb deficit –duration product was useful in predicting survivors (162 g/L x days, 130 g/L x days; median, interquartile range) vs. non-survivors (211 g/L x days, 298 g/L x days; $p = 0039$) and this may be a more meaningful parameter for future evaluations.

Despite the limitations of a "Hb transfusion trigger", a few studies correlated Hb concentration to patient outcome and these studies refute the criticisms of the Hb trigger chosen in these OT cases. First, Hb levels of 50 g/L were reported to be tolerated without evidence of organ hypoxia in *healthy* volunteers (Weiskopf 1998) but Hb concentrations of $\sim$ 25 g/L or less were associated with mortality (Weiskopf 2010a). Combined with our knowledge of the impact that co-morbidities have on ischemic events, the maximum tolerated Hb trigger of 50 g/L would seem reasonable for a sick or injured patient. The correlation between Hb and the in-hospital post-operative 30-day mortality, the ultimate endpoint, for 300 Jehovah's Witness patients undergoing major non-cardiac surgery shows a clear continuum with a large difference in percent survival between Hb concentrations of $> 51$ g/L and $\leq 50$ g/L (Table 31.4, Carson 2002). As a comparison, the survival and nadir Hb of the OT CU cases is also provided, showing a similar trend (Fig 31.1) but higher survival at very low Hb ($< 30$ g/L) concentrations which suggests a survival advantage with an OT.

**Table 31.4** Comparison of lowest patient hemoglobin concentration and survival

| Nadir Hb (g/L) | Patients refusing blood (Carson 2002) % Survival (n) | OT CU cases % Survival (n) |
|---|---|---|
| 11–20 | 0 (7) | 29 (7) |
| 21–30 | 46 (24) | 59 (17) |
| 31–40 | 75 (28) | 36 (22) |
| 41–50 | 66 (32) | 82 (11) |
| 51–60 | 91 (54) | 43 (7) |
| 61–70 | 91 (56) | 75 (4) |
| >70 | 100 (99) | No cases with this Hb |

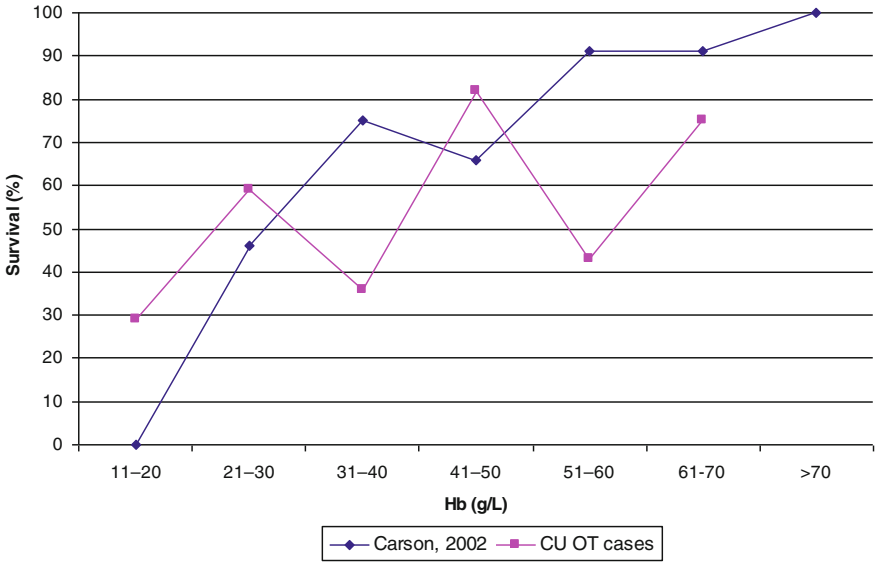


**Fig. 31.1** Percentage patient survival based on nadir hemoglobin concentration for 300 Jehovah's Witness patients undergoing major noncardiac surgery (Labeled as Carson 2002) and for 69 compassionate use oxygen therapeutic patients (Labeled as CU OT cases)

## 31.7 Risk:Benefit of Oxygen Therapeutic in Individual Compassionate Use Cases

Some of the individual case reports provide a detailed look, within a particular organ system or clinical scenario, at the risk:benefit of this drug class. A few of these cases illustrate unexpected results and show that the concerns over theoretical risks are not always borne out clinically. For example, there appear to be intrinsic side-effects of HBOC compounds, including blood pressure elevation, abdominal pain, jaundice, and elevations in liver, pancreatic and cardiac enzymes. These side effects, provided by clinical trial results, have been mostly classified as minimal to mild. However, some studies have shown marked elevations in blood pressure and others have reported increased evidence of troponin leak without clinical signs of myocardial infarctions ("micro-infarcts"). The mechanism of these reactions, particularly the blood pressure elevations, is thought to be scavenging of nitric oxide by free hemoglobin (Winslow 2007; Greenburg 2004). These safety concerns have been raised by FDA and have led to a "clinical hold" of many HBOC trials in the USA (Auker 2011; Natanson 2008). However, several of the individual OT CU cases (Table 31.1), were actually administered in situations where a clinical trial would not have been approved precisely because of these known or perceived adverse events associated with the drug. In other cases, data is provided to the contrary of the expected adverse event and the HBOC was

considered to be beneficial. A few such examples are provided in a description of the individual cases.

## 31.8 Does Blood Pressure Increase with an Oxygen Therapeutic?

With regards to the blood pressure elevations, both systemic and pulmonary blood pressure increases have been described in preclinical models as a rapid elevation that peaks within minutes of the HBOC administration and then plateaus before a gradual return to baseline. The dose required is a very small, subclinical dose because only a small amount of free hemoglobin is needed to cause a decrease in circulating nitric oxide. Thus in all of these CU cases, blood pressure elevations would be expected to occur immediately and should be identified in the medical records. In the MacKenzie series, systolic blood pressure increased (>160 mm Hg) in 19 of 39 patients, with eight patients (three of 10 survivors and five of nine non-survivors) requiring antihypertensive drugs. The other OT CU cases reported that all three of the hemoglobin raffimer CU patients had elevations in blood pressure following the OT while there was no blood pressure elevation related to the OT for the nine patients who received HBOC-201 or Polyheme and for which data was provided (Table 31.1). One of these nine patients developed a severe increase in SBP 12 h after the last HBOC dose which was not attributed to the OT (Marinaro 2009). This was a TBI patient where critical hypertension (SBP of 280 mm Hg) occurred and, given the delay in it's development, was more likely due to a progression of the brain injury itself (brain herniation) than a direct vasoconstrictive or hypertensive effect of the HBOC. The authors commented that the patient's death may have been from massive reperfusion injury due to delayed repayment of severe cerebral oxygen debt (Marinaro, 2009).

These data seem to indicate that OTs are not equal in their effects on blood pressure. HBOC OTs with higher tetrameric and/or dimeric hemoglobin have higher systemic vasoconstrictive activity and cause more potent blood pressure effects (Rice 2008). Older aged subjects in clinical trials also seem to respond with higher blood pressure responses (Freilich 2009). Hemoglobin raffimer showed a consistent three out of three patients with an increase in blood pressure, including one patient who developed pulmonary hypertension (Shander 2004). On the other hand, neither of the other two HBOCs showed consistent increases in blood pressure and this latter patient is the only patient in which *pulmonary* hypertension was specifically noted (either because this was a rare occurrence or because patients did not have pulmonary pressures monitored). In fact, a few case reports noted a resolution of hypotension and ability for the patient to be weaned from vasopressor support following the OT therapy (Mullen 2000; Gannon 2002; Stefan 2007; Pachinburavan 2008). Clearly, increases in blood pressure following OT administration are dependent upon many factors that influence the medical condition of the patient and, when used properly, may be more of a "benefit" than a "risk".

## 31.9 Do Oxygen Therapeutics Cause Myocardial Toxicity?

Similarly, the "risk:benefit" of OT on the myocardium is another area of medical controversy. Based on the adverse cardiac events in clinical trials using different HBOC compounds, myocardial injury ("toxicity") is currently considered to be a major safety concern of HBOC administration. This is thought to be a class effect of these drugs (Silverman 2009; Natanson 2008). It may also be that humans exhibit a species-specific sensitivity to the cardiac effects of HBOCs (Silverman 2009; Natanson 2008; and Burhop 2004). This makes preclinical evaluation of new OT particularly challenging as negative results in animal models may therefore not predict safety in humans and, if the risk outweighs the benefit, clinical trials to evaluate cardiac effects would not be allowed by federal agencies. This is a situation where OT CU cases may assist in testing the medical hypothesis of whether or not HBOCs are cardiotoxic. There are a few individual case reports that seemingly demonstrate an HBOC showing myocardial protection through either direct or indirect evidence.

In the first case, a 32-yr-old Jehovah Witness woman was involved in a life-threatening motor vehicle accident. Her spinal cord was nearly severed, her lungs collapsed, skull fractured, ribs, cheekbone and an elbow broken and her spleen was ruptured (Fitzgerald 2011). Troponin I levels were carefully measured throughout the patient's hospital stay. Concentrations increased dramatically from 20 ug/L (Day 3) to $\sim$ 53 ug/L (Day 4) peaking at $\sim$ 75 ug/L (Day 5). At this time (Days 5 and 6), HBOC-201 was administered and troponin levels declined to $\sim$ 30 ug/L (Day 7) and then to less than 10 ug/L by Day 10. Electrocardiogram (ECG) changes compatible with myocardial ischemia were correlated with the troponin changes and resolved with OT intervention. Thus, these results are at odds with the FDA concern of "subclinical myocardial infarctions" based on mildly elevated troponin levels in South African patients in the HBOC-201 HEM-0115 trial (Jahr 2008). However, those troponin levels were sporadically taken during the trial with rarely more than two readings for any particular patient, results were not consistent across patients, and there was no correlation between these troponin "leaks" and serious adverse events. On the other hand, this single CU case, where the physicians were aware of potential cardiac risks and sought to monitor the patient closely, is, to date, the clearest documentation of troponin levels in a human after HBOC-201 administration. One may argue that a subclinical myocardial toxic effect of the OT could have been overshadowed by the troponin increases from cardiac ischemia, but this is precisely the type of critical case in which the theoretical risk of HBOC administration is greatly outweighed by its benefit.

Although not a CU case, another case report provides additional support for the concept that HBOCs can be cardioprotective. This case report is of a patient who was enrolled in an HBOC clinical trial (Nequille 2000). This report described a 64-yr-old patient, with a history of cardiac disease, who was scheduled to undergo an aortobifemoral graft revision and who also was enrolled in a randomized, single-blinded HBOC trial in the event of intraoperative anemia. Intraoperatively, ST-T

segment depression developed and, at a Hb of 7.6 g/dL, the decision for a transfusion was made. Critically, during the infusion of the first 500 ml of HBOC-201, the authors observed rapid normalization of the ECG directly attributable to the HBOC. Then, "during the postoperative period, recurrent tachycardia and ST-T changes were successfully treated by a second infusion of HBOC-201" showing that although the original intention was to use this HBOC as a substitute for blood, the second dose was, instead, for the treatment of myocardial ischemia.

In the third study, HBOC-201 was infused in a child with sickle cell disease who was also in cardiac failure (Stefan 2007). This report was from South Africa, where the product was already registered for use in adults but was administered to the child as an off-label use. At the time, there was no information available about the product in children; all clinical trials were done using adults with acute surgical anemia. Further, in South Africa, it is not approved for use in sickle cell anemia although the company had performed a Phase I/II trial in adults with sickle cell (Gonzalez P 1997). Typically, this situation would have been managed with an early transfusion of blood but HBOC-201 was used in this case, in place of blood, because of the family's religious preferences. The OT was able to augment the oxygen-carrying capacity of the blood, improve cardiac perfusion and reverse the ischemic cardiomyopathy associated with the sickle cell crisis.

Clearly these three cases do not provide enough evidence to completely refute concerns over possible myocardial injury due to HBOC administration, but they do suggest that individual patient conditions may result in a cardiac benefit, rather than a cardiac risk. A *carte blanche* conclusion that HBOCs are "cardiotoxic" does not necessarily reflect their true risk:benefit profile and may result in unnecessary delays in regulatory approval. In these individual patients, cardiac ischemia was resolved with an HBOC, suggesting that HBOCs, as a class, may be beneficial in the very patients about whom there is concern. In one preclinical study in severe hemorrhagic shock, myocardial histopathology was similar or in some cases better with HBOC-201 resuscitation than with standard fluid (Johnson 2006) while in other preclinical studies of myocardial infarction, treatment with HBOCs reduced myocardial infarct size (Rempf 2009; George 2006; Caswell,2005). Moreover, the results of a retrospective analysis of subpopulations of patients treated with HBOC-201 suggested that risk of cardiac adverse events was age-related; thus, some patients may have higher risk than others for potential cardiac toxicity (Freilich 2009). While HBOC critics often site a meta-analysis of HBOC trial data that allegedly "proved" an increase in the incidence of myocardial infarctions and death in patients who were anemic and received an OT (Natanson 2008), that conclusion has recently been disputed (Keipert 2008; Levien 2008; Shander 2008; Sauala 2008). If humans are, indeed, particularly sensitive to cardiac effects of HBOCs, then resolving this issue of cardiotoxicity will be quite challenging and these CU cases may provide insight into how to develop safe clinical trials to evaluate this risk.

## 31.10 Do Oxygen Therapeutics Cause Cerebral Toxicity?

Despite numerous preclinical studies of brain injury indicating potential benefit of HBOC treatment, traumatic brain injury (TBI) was a specific exclusion criterion of the Polyheme trial, the most recent HBOC clinical trauma trial (Gould 2002). The effect OTs may have on ischemic brain tissue is complex, as illustrated in the recent case report in which a 21-yr-old male Jehovah Witness was hit and dragged by a motor vehicle, causing severe TBI and soft tissue injury. Six days after injury the patient had a Hb of 3.5 g/dL and the CU process was initiated. The OT was administered on Day 7 with physicians noting, as one of the most notable changes after the first HBOC infusion, a bilateral improvement in regional cerebral oxygenation ($BrSvO_2$) as measured by a cerebral oximeter and also normalization of mixed venous oxygen saturation ($SvO_2$). The right cerebral hemisphere, the most injured side, began at a lower baseline ($BrSvO_2$, 25 %; normal, 50 %-70 %) and normalized ($BrSvO_2$, 54 %) after the OT infusion. In addition, creatinine levels and heart rate improved. Transient acute hypertension was not seen after OT infusion although, as discussed earlier, the patient had a hypertensive event (systolic to 280 mm Hg) 12 h after the last OT infusion. At that time, signs and symptoms deteriorated with the clinical and radiographic evidence being consistent with acute cerebral herniation. Life support was withdrawn on Day 10 with the patient quickly expiring. This case report is remarkable in that it demonstrated improved cerebral oxygenation with an HBOC in the face of severe TBI and when not administered until nearly 8 days from the time of head injury. It is possible that the delayed administration of the HBOC may have altered the final outcome but this cannot be determined from the case. It cannot be determined whether or not the HBOC itself directly caused the cerebral edema and herniation, despite the improvement in cerebral oxygenation, nor can it be determined if herniation could have been prevented if the OT had been administered sooner, at a different dose or rate or with concurrent other medications. The authors commented that the patient's death may have been from massive reperfusion injury due to delayed repayment of severe cerebral oxygen debt. The only other study that involved patients with intracranial events was an article that evaluated a first generation HBOC, diaspirin cross-linked hemoglobin (DCLHb; Baxter Healthcare Corp., Deerfield, IL) in patients after acute ischemic stroke (Saxena R 1999). The authors found worse outcome scores and two adverse events possibly related to the administration of the OT. One event involved a patient with acute cerebral edema followed by death which is similar to the timeline of the current TBI patient. These reports raise conflicting possibilities that HBOC treatment of TBI may be either beneficial or harmful. That HBOC-201 has shown improvements in contralateral brain oxygenation levels and histopathology in preclinical models of hemorrhagic shock with traumatic brain injury (polytrauma) provides a suggestion that similar effects could occur with ischemic stroke and other cerebrovascular accidents (Stern 2009). The possibility that cerebral edema and herniation may result from delayed re-perfusion injury offers a potential unifying concept and a testable

hypothesis. If true, it may be that the timing of administration of an HBOC may be crucial to the benefit:risk balance.

## 31.11 Conclusions

The life-supporting qualities of oxygen therapeutics should no longer be open for debate. These CU cases clearly show that there are patients where the very real risks associated with their low hemoglobin levels outweigh the potential risks of OTs. OT may be life sustaining in patients who do not have immediate access to red cells, acting as a bridge to endogenous production or red cell transfusion; additionally, as evidenced in many of the CU patients, OT may be life saving in individuals who do not accept RBC transfusions. It is agreed these case report testimonials do not provide the evidence necessary for OT approval by regulatory authorities. These cases do, however, provide physicians with examples of what the future may one day bring: an OT fluid, compatible with all blood types, which will provide a temporary oxygen bridge from symptomatic anemia when blood is not an option. For Jehovah Witness's and similar groups that refuse or cannot accept blood, these CU cases may well be the spark that keeps the fire alive for the day when there is an accessible alternative to an RBC transfusion.

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# Part VIII
# HBOC-Mediated Adverse Effects

# Chapter 32
# Acellular Hemoglobin-Based Oxygen Carrier Mediated Blood Pressure Elevation and Vasoconstriction: A Review of Proposed Mechanisms and Contributing Factors

**Hae Won Kim**

## 32.1 Introduction

Hemoglobin-based oxygen carriers (HBOCs) are in advanced stages of clinical development as potential red blood cell substitutes ('blood substitutes') for treatment of acute anemia due to traumatic or surgical hemorrhage and other conditions (Kim 2004b, 2006; Jahr 2011). Recently, phase III clinical studies of some leading candidate HBOCs have been conducted but were not successful obtaining regulatory approval due to failure to achieve predetermined endpoints and concerns about unfavorable safety profiles (Moore 2009b, Jahr 2012, Bernard 2011). In the spring of 2008, a NIH-FDA sponsored workshop was held to address the safety issues of HBOCs and propose recommendations for future directions (Silverman 2009). There was a major concern about the propensity of acellular HBOCs to elicit blood pressure elevation because it was due primarily to systemic and pulmonary vasoconstrictions. HBOC-mediated vasoconstriction could cause suboptimal blood flow to critical organs leading to organ dysfunction and failure.

Some HBOC producers have claimed that their products are non-vasoactive (Johnson 1998; Vandegriff 2003). However, FDA recently stated that all HBOC products it reviewed, regardless of structure and molecular weight distribution, were vasoactive at the doses proposed for clinical use (FDA 2004; Silverman 2009). The HBOC-mediated hypertensive effect (BP elevations) observed appears to occur primarily due to systemic vasoconstriction as they occur without concomitant increase in the cardiac output. For example, in patients undergoing aortic surgery or percutaneous coronary procedure, administration with HBOC-201

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(OPK Biotech/Biopure, Cambridge, MA) increased systemic and pulmonary pressures and vascular resistance (Kasper 1996, 1998; Serruys 2008). In most cases, HBOC administration elicited a dose dependent mild to moderate increase in systemic BP which spontaneously resolved within few hours or following anti-hypertension medications (Greenburg 2004; Freilich 2009). However, incidences of severe hypertensive events (>180 mm Hg systolic BP) have been observed in patients with existing hypertension and other co-morbidities (Serruys 2008). The mechanism(s) of HBOC-mediated BP elevation/vasoconstriction has not been fully elucidated and its clinical consequences have not been identified. If HBOC administration elicits vasoconstriction severe enough to cause ischemic conditions in key organs, organ dysfunction and failure could result. In fact, a recent study asserts that HBOCs increased risk of myocardial infarction and death based on a meta analysis of past HBOC trial results (Natanson 2008). This study is highly controversial because its conclusions were based on pooled data from 16 trials involving 5 chemically distinct products and 3,711 patients with various clinical conditions. There were questions regarding the accuracy of data and validity of study methodology (Fergusson 2008; Levien 2008; Keipert 2008; Lewis 2008; Sarani 2008; Sauaia 2008). Nonetheless, the etiology and clinical ramifications of HBOC-associated serious adverse events must be fully addressed before regulatory approval of these products can be obtained (FDA 2004, 2008).

In this chapter, to help elucidate the etiology of the adverse effects of HBOCs, key currently proposed mechanisms for the HBOC-mediated vasoconstriction/BP elevation will be reviewed. In addition, some key factors that contribute to overall manifestation of HBOC-vasoactivity will be discussed.

## 32.2 Proposed Mechanisms for the HBOC-Mediated BP Elevation and Vasoactivity

A physiologic saline solution of 'purified' human Hb obtained by hypotonic rupture of washed red blood cells was tested as early as early 1910s in animals and human subjects mostly without acute reactions. Hb infusion in amounts sufficient to cause hemoglobinuria did not cause any notable toxicity in normal subjects but caused temporary loss of consciousness and hemolysis in a patient with advanced pernicious anemia which improved after 1.5 h after excretion of Hb (Sellards and Minot 1916, 1917). In late 1940s, (Amberson1947, Amberson et al. 1949) reported perhaps the first observation of pressor/hypertensive responses in human subjects following intravenous Hb administration but without acute ill effects (e.g., anaphylactic reaction, hemolysis) although there were some undesirable reactions including mild chills and oliguria. They attributed the adverse effects to possible bacterial contamination of test Hb solution, denatured or toxic derivatives of Hb, red cell membrane stroma and other deleterious red cell constituents (e.g., enzymes) that passed through the purification. Later, Rabiner et al. (1967), asserting that the renal toxicity observed with Amberson's Hb solution was due to

erythrocyte membrane stromal residues that remain in their Hb solution, developed a new method to prepare 'stroma-free' Hb (SFH) solution. However, this SFH solution was also found to elicit pressor effect when tested in healthy young healthy volunteers (Savitsky 1978). Subsequently, higher purity hemoglobin solutions prepared by modern chromatographic methods still exhibited vasopressor effect in isolated organ studies (Kim 1983, 1988; Macdonald et al. 1990, Macdonald and Motterlini 1994; Vogel 1986). Since then, despite extensive purification followed by chemical modifications, most human or bovine Hb derived HBOCs developed as potential red blood cell substitute have been shown to elicit pressor effects in preclinical and clinical studies (Silverman 2009). In the meantime, it was discovered that the vascular endothelium constitutively produces gaseous nitric oxide (NO) as a potent vascular relaxation factor (Martin 1985, 1986; Moncada 1994, Furchgott 1989; Ignarro 1989). Interestingly, in 1950s, Gibson et al. had already found that NO avidly binds sheep Hb with very high affinity (1955, 1957). Since then, it has become the most widely accepted hypothesis that acellular Hb/HBOC scavenging of NO is a principal mechanism in the Hb/HBOC-mediated BP elevations/vasoconstriction (Kim 1995, 1997; Gulati 1997; Moisan 1998; Freas 1995; Muldoon 1996; Pawson 2007). However, NO scavenging by Hb/HBOC alone does not satisfactorily explain all the abnormal hypertensive effects observed in certain HBOC studies. Therefore, other mechanisms have also been proposed to participate in the genesis of HBOC-mediated pressor responses (Moore 2009a).

### *32.2.1 Acellular Hb/HBOC Scavenging of Endothelial NO (EDNO)*

Normal arterial vascular tone is regulated by endothelium-derived NO, a potent vasodilator constitutively produced in the vascular endothelium by actions of NO synthase (eNOS) (Martin 1985, 1986). NOS converts L-arginine to NO and citrulline. Normally, bulk of basal endothelial NO produced is directed toward the smooth muscle, an effector organ located abluminal side of the endothelium. Once in the vascular smooth muscle (VSM), NO activates soluble guanyl cyclase (sGC) to produce cGMP which mediates myosin light chain (MLC) dephosphorylation resulting in weaker actin-myosin coupling (vascular relaxation). Although NO has an avid affinity for ferrous Hb ($2–6 \times 10^7$/M/s, Cassoly and Gibson 1975), its reaction with Hb contained in the red blood cells (RBCs) is limited because RBC membrane serves as a barrier. In addition, in arterioles or larger blood vessels, RBCs generally flow along the center forming a 'cell free' zone near the endothelium surface thereby making reaction between RBC-Hb and endothelial NO more difficult. The cell-free or acellular Hb/HBOC in blood is much more reactive to endothelium derived NO because there is no diffusion barrier that separates them. In addition, unmodified Hb tetramers/dimmers that may remain in the HBOC products or smaller molecular weight HBOC products could easily

extravasate through the endothelial fenestrations into subendothelial space where they could readily scavenge NO during its passage to the smooth muscle. In vessels with more porous membranes (e.g., hepatic vein/sinus) or in pathologic conditions that cause increase in endothelial permeability (e.g., inflammation, sepsis), even smaller MW polymerized HBOCs may extravasate. Under those conditions, NO levels in VSM would be reduced resulting in lower cGMP level to inhibit myosin light chain kinase (MLCK) activation. This, in turn, results in increased myosin phosphorylation and actin binding thereby shifting the VSM toward a more contracted state (vasoconstriction). This process is summarized in Fig. 32.1.

There are two main reactions known to occur in blood between cell-free Hb and NO:

(1) $HbO_2 + NO \rightarrow metHb + NO_2^-/NO_3^-$ (in arterial blood)
(2) $Hb + NO \rightarrow HbNO$ (nitrosyl Hb) (in venous blood)

In arterial blood, Hb is predominantly in the oxygenated state ($HbO_2$). When $HbO_2$ ($Fe^{+2}$) reacts with NO, Hb is oxidized to ferric ($Fe^{+3}$) Hb (metHb) and NO is converted to nitrate or nitrite (reaction 1 above). In venous blood where $PO_2$ is lower, a substantial portion of Hb is in the deoxygenated state (Hb). Reaction of deoxy Hb with NO results in formation of iron nitrosylated Hb (HbNO) (reaction 2

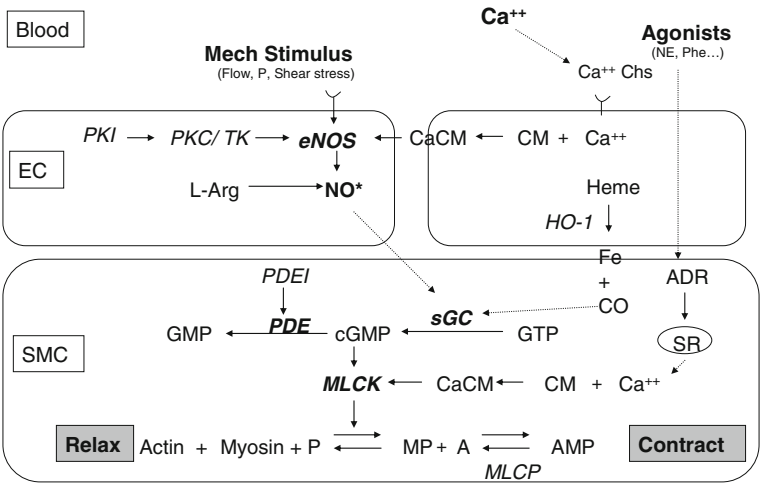


**Fig. 32.1** Role of endothelial NO in the vascular smooth muscle contraction/relaxation. EC: endothelial cell, SMC: vascular smooth muscle cell, AchR: acetylcholine receptor, $Ca^{++}$Chs: calcium channels, PKI: protein kinase inhibitor, PKC: protein kinase C, TK: tyrosine kinase, CM: calmodulin, PDE: phosphodiesterase, PDEI: phosphodiesterase inhibitor, SR: sarcoplasmic reticulum, ADR: adreno receptor, MLCK: myosin light chain kinase, MLCP: myosin light chain phosphatase, MP: phosphorylated myosin. AMP: phosphorylated myosin bound to actin

above). In addition, to a less extent, reactive cystienes of $\beta$-globins ($\beta$93Cys) have been proposed to react with NO to produce S-nitroso Hb which is proposed to play a role in vasodilation of ischemic/hypoxic blood vessels (Jia 1996). Interestingly, recently Hb is also proposed as a nitrite reductase which converts plasma nitrite into NO under certain favorable conditions (Gladwin 2008). Then, the overall effect of Hb/HBOC on the vasoactivity will depend on the net NO balance (endothelial NO production + NO formation by nitrite reduction – NO scavenging).

To confirm that infusion of exogenous Hb into blood can actually react with endogenous NO, a small amount of purified human Hb was infused into the blood vessel of the endotoxemic rats, a condition that is known to produce a higher level of NO by activation of an inducible isoform of NOS (iNOS). Plasma samples were collected and analyzed for nitrosyl Hb (HbNO) formation, evidence that Hb reacted with NO. Indeed, HbNO formation in the plasma samples (where the infused Hb is present) was confirmed by EPR spectroscopy (Greenburg 1995; Kim 1995).

In addition, while vessel rings with intact functional endothelium Hb elicited contraction, Hb did not cause contraction in vessel rings whose endothelium was mechanically or chemically removed (Kim 1995, 1997, 2000). Similarly, in vessel rings with intact endothelium but pretreated with L-NAME ($N^G$-nitro-L-arginine methyl ester; NOS inhibitor), Hb did not elicit significant contraction (Kim and Greenburg 1997).

In isolated vessel segments from various species (rat, rabbit, dog, pig, bovine, etc.) and different vessel phenotypes (thoracic aorta, carotid artery, umbilical vein, mesenteric artery, coronary artery, etc.), acellular HBOCs elicit significant additional contractions generally in vessels precontracted with an agonist (Kim 1995; Freas 1995; Muldoon 1996; Jing 1996; Nakai 1996; Hart 1997; Abbasi 1997; Pawson 2007). In addition, decreased cGMP levels were observed following DCLHb treatment, an indication of NO levels that activate soluble guanyl cyclase (Gulati 1997), supports the Hb scavenging of NO hypothesis. The DCLHb-induced contractile responses were greatly attenuated by L-NAME or endothelial removal while not significantly affected by BQ-123 ($ET_A$ receptor antagonist), prazosin (alpha 1 receptor antagonist) or indomethacin (cyclooxygenase inhibitor). In rat mesenteric and human radial arteries, DCLHb effectively abolished carbachol induced relaxation while it had no effect on endothelium-derived hyperpolarizing factor (EDHF)-mediated component (Vuylsteke 2001).

Mathematical modeling studies have also been conducted to analyze HBOC scavenging of NO in the microcirculation by modeling NO diffusion and consumption in an arteriole when HBOC is present (Kavdia 2002; Tsoukias 2008; Gundersen 2009; Jeffers 2006). They concluded that HBOC would significantly reduce NO levels near the vascular wall (vascular smooth muscle). It was shown that Hb as low as 1 μM significantly reduced NO bioavailability and Hb extravasation elicited even greater effect (Jeffers 2006). This is consistent with previous experimental observation that threshold concentration of cell-free Hb for

contraction of isolated rat thoracic aortic ring preparations was nanomolar range (Kim and Greenburg 1997, 2000).

Taken together, it appears that Hb inactivation of endothelial NO is a primary player in the Hb/HBOC-mediated vasoconstriction.

### 32.2.2 $PO_2$ Dependent Vascular Autoregulatory Hypothesis

If NO scavenging is the sole mechanism for the HBOC-mediated pressor effects, a HBOC with a higher NO binding affinity should elicit stronger pressor effect than one with a lower NO affinity. To test the hypothesis, NO reactivities of three HBOCs with varying degree of vasopressor effects (HBOC-301, αα-crosslinked Hb, PEG-Hb) were assessed in rats following 50 % isovolemic exchange transfusion (Rohlfs 1998). Surprisingly, HBOC preparations that elicited transient or no significant BP elevation had a stronger NO binding affinity than that elicited sustained BP elevations. In hemorrhagic shocked swine, resuscitation with HBOC did not elicit vasoconstriction nor BP elevation (Fitzpatrick 2004).

Additionally, low $O_2$ affinity HBOCs were shown to elicit decreased functional capillary density in a hamster skinfold microcirculation model compared with HBOCs with a high $O_2$ affinity or non-oxygen carrying control solution (Tsai 2003). Based on this observation, an alternative hypothesis has been proposed that HBOCs with a lower than normal $O_2$ affinity may lead to premature oxygen offloading in the arterioles upstream from the capillaries where $O_2$ supposed to be unloaded. Such premature unloading of $O_2$ could cause overoxygenation and trigger reactive arteriolar vasoconstriction resulting in decreased functional capillary density ($PO_2$ dependent autoregulatory vasoconstriction) (Vandegriff 2003). Matheson, et al. (2002) also observed that a low $O_2$ affinity ultra high molecular weight HBOC caused cerebral arteriolar vasoconstriction in rats and cats. They theorized that the elevated $PO_2$ as a result of premature $O_2$ offloading in the arterioles would stimulate 20-HETE production which causes arteriolar constriction to prevent over-oxygenation (Qin 2006).

Based on the autoregulatory vasoconstriction theory, MP4, a 'non-vasoactive' HBOC with a high oxygen affinity (Hemospan®, P50 = 6 mm Hg, Sangart Corp.) was developed (Vandegriff 2003; Tsai 2003). This PEG conjugated Hb based product does appear to have reduced pressor effect compared with DCLHb in rats. However, it should be noted that MP4 contains only 4 g/dl of Hb compared with 10 g/dl in DCLHb making it difficult for direct comparison. In another study with swine subjected to hemorrhagic shock, resuscitation with MP4 caused a statistically insignificant increase in MAP but it did elicit a significantly increased pulmonary arterial BP and vascular resistance (Drobin 2004) which is in contradiction to the assertion that MP4 is non-vasoactive.

Using a hamster window chamber model, Tsai et al. (2006) measured changes in macro and microvascular hemodynamic parameters and perivascular NO levels following 10 % topload infusion of α-α crosslinked Hb, bovine polymerized Hb

(HBOC-301, Biopure/OPK Biotech), and PEG-Hb (MP4). They observed that all three Hb preparations caused MBP increases and reduction in perivascular NO levels. However, unlike the other two Hb treated groups, in the MP4 infused animals, reduction in microvascular diameter and blood flow did not occur. Based on this finding, they claimed that microvascular responses to MP4 is likely to involve factors besides NO scavenging such as blood volume expansion due to high COP of PEG-Hb (70 mm Hg) (Vandegriff 2009). However, it should be noted that measurements of peripheral skin microcirculatory changes may not properly represent microcirculatory status of critical organs such as the brain, hearts, lungs and kidneys.

Although it appears to work in certain vascular beds (e.g., cerebral arteries), universal applicability of the $PO_2$ dependent autoregulatory vasoconstriction theory for the HBOC-mediated BP elevation/vasoactivity has been controversial. For instance, some high $O_2$ affinity HBOCs were also shown to elicit vasoconstriction/BP elevation while a low $O_2$ affinity HBOC preparation were shown to better improve tissue oxygenation than one with a high $O_2$ affinity (Cabrales 2010). More studies are needed to test its validity and universal applicability to other blood vessel types.

### *32.2.3 Hb stimulation of Endothelin-1*

It has been proposed that ET-1 may play a significant role in Hb-mediated vasoactivity (Rioux 1999). Endothelin-1 (ET-1) is one of three endothelins, a family of peptides of 21 amino acids. Although endothelins are produced by variety of cell types including vascular smooth-muscle cells, ET-1 is the only endothelin subtype that is produced in the vascular endothelial cells. Hypoxia, ischemia and shear stress induce transcription of ET-1 mRNA leading to synthesis and secretion of ET-1. Vascular cells can rapidly adjust ET-1 production as required for the regulation of vasomotor tone. All three endothelins bind to two types of receptors, Endothelin-A or B receptors ($ET_AR$ or $ET_BR$), expressed on the cells of many mammalian species including humans. Endothelin-A receptors have a high binding affinity for ET-1 and are expressed abundantly on vascular smooth-muscle cells and cardiac myocytes. These receptors mediate the vasoconstrictor action of ET-1. The activated endothelin receptors stimulate activities of phospholipase C which produces inositol 1,4,5-triphosphate and diacylglycerol. The former increases the intracellular calcium concentration, which in turn causes the vasoconstriction. The vasoconstriction mediated by ET-1 persists even after ET-1 is removed from the receptor, probably because the intracellular calcium concentration remains elevated. Nitric oxide shortens the duration of vasoconstriction by facilitating restoration of intracellular calcium to its basal level (Levin 1995). Vascular endothelial cells express $ET_BR$ which upon activation release NO and dilate vessels via GC/cGMP-mediated mechanism thus counter balancing ET-1/$ET_AR$ mediated vasoconstriction (Dhaum 2008). Therefore, it is plausible that

reduction in NO by HBOC could lead to shifting the vasoconstriction/dilation balance to vasoconstriction as $ET_BR$ mediated vasodilatory influence is reduced. This could explain why HBOC caused elevated BP in diabetic mice but not in eNOS knock-out mice (Yu 2010).

Gulati et al. (1995) reported that intravenous administration of DCLHb increased ET-1 level in animal studies. In hemorrhagic shocked rats, administration of 400 mg/kg DCLHb significantly improved systemic hemodynamics, blood flow, $O_2$ consumption and base deficit and extended survival time. Hemorrhage caused increases in both ET-1 and cGMP levels. In contrast, resuscitation with DCLHb increased ET-1 levels while decreasing the cGMP level. Pretreatment with L-NAME or FR-139317 (an $ET_AR$ antagonist) attenuated hemodynamic and beneficial effects of DCLHb (Gulati 1997). The DCLHb-mediated increase in ET levels was reported to occur through a mechanism other than stimulation of ET converting enzyme because treatment with phosphoramidon, an inhibitor of pro-endothelin to ET converting enzyme, did not attenuate the cardiovascular effects of DCLHb (Gulati 1995, 1997). This observation appears to contradict earlier findings by Schultz et al. (1993) who reported that phosphoramidon attenuated DCLHb-mediated pressor effect. In addition, BQ-123, another $ET_AR$ antagonist, attenuated hemodyanmic effects of DCLHb (Gulati et al. 1996). However, in isolated rat aortic vessels, pretreatment with BQ-123 did not prevent Hb-induced contraction suggesting that ET-1 may have limited role in this model (Tai 1997). Further, in a study of human endothelial cells, Hb directly inhibited ET-1 production and secretion (Simoni 1995). Nevertheless, in a Phase III clinical trial of ischemic stroke, infusion of DCLHb was associated with a dose-dependent increase in plasma ET-1 concentration (Saxena 1998). It appears that there is no clear consensus on the role of ET-1 in the Hb-mediated BP elevation/vasoconstriction. It is plausible that, ET-1 mediated contraction occurs with certain types of HBOCs (e.g., DCLHb) or in selected type of blood vessels and/or animal models. Further studies are needed to clarify the role of ET-1 in HBOC-mediated vasoactivity.

### *32.2.4 Hb Stimulation of α-Adrenergic Receptor*

Administration of HBOCs may stimulate central or peripheral sympathetic nerves that increase vascular tone. To investigate this hypothesis, DCLHb was administered to cervically sectioned rats. In these animals, DCLHb administration increased BP which was comparable to that observed in normal rats. In cervical sectioned rats, DCLHb potentiated clonidine (presynaptic α-2 adrenergic receptor agonist) induced pressor effect (Gulati 1994a, b). This indicates that the DCLHb mediated pressor effect was mediated through peripheral mechanisms rather than the central nervous system. Further, DCLHb also elicited pressor effect in bilaterally adrenal demedullated rats indicating that the pressor effect was not mediated by catecholamines or other vasopressor mediators released by adrenal medulla.

Pretreatment with DCLHb markedly potentiated pressor effects of norepinephrine, phenylephrine and clonidine. Clonidine lowers BP in normal animals through a centrally acting negative feedback mechanism that results in lowered sympathetic output. But, in cervical sectioned rats, it elicits pressor effect which was potentiated by DCLHb. This potentiation was attenuated by prazosin (α-1 antagonist) and yohimbine (α-2 antagonist) indicating α-adrenergic involvement (Gulati 1994b, Sharma 1995).

In an isolated rat aortic model where there is no CNS influence, Hb-mediated contraction occurs only when the vessel is first precontracted with an agonist typically with an α-adrenergic agonist such as norepinephrine, phenyleprhine. This raises a question that whether a local adrenergic activation may be required for the Hb mediated contraction to occur. This was investigated by Kim et al. (2001a, 2004a, 2005a). Inhibition of alpha adrenergic receptors by phentolamine prevented Hb induced contraction. However, in the presence of phentolamine, precontraction with non-adrenergic agonists (e.g., serotonine, PGF2α, KCl) allowed Hb induced contractions. These results suggest that, although not a prerequisite, local adrenergic influence may play a key role in HBOC-mediated contraction because sympathetic tone is usually active under normal physiologic conditions. It is hypothesized that precontraction of vessels with an agonist activates mechanosensitive NOS present in the endothelium releasing NO as a part of dynamic homeostatic mechanisms to counterbalance the vascular tone (Kim 2001c). Thereby, subsequent treatment with Hb/HBOC allows further contraction as it tips the balance toward more contraction by inactivating NO. That pretreatment with L-NAME, a NOS inhibitor, abolished the HBOC-mediated additional contraction support the hypothesis.

### *32.2.5 Hb-Mediated Production of Toxic Redox Products*

Hemoglobin can function as an $O_2$ carrier only in the ferrous ($Fe^{2+}$) heme form. This functional ferrous Hb can be oxidized to non-$O_2$ carrying ferric ($Fe^{3+}$) Hb (metHb) and ferryl ($Fe^{4+}$) Hb derivatives under oxidizing conditions. Also, metHb can form via autooxidation of ferrous Hb that produces superoxides in the process. A more stable ferryl ($Fe^{4+}$) Hb can form when cell-free Hb is allowed to undergo a further oxidation by treatment with peroxides (e.g., $H_2O_2$, lipid peroxides, peroxynitrites).

This ferryl Hb is highly cytotoxic that can cause cellular apoptosis and death. Therefore, ferryl Hb, especially in the presence of inflammatory condition, could cause damage to the vascular endothelial and smooth muscle cells (Alayash 2001a, Alayash et al. 2001b, c, D'Agnillo 2000). Damaged vessels, in turn, could release more vasoactive mediators and procoagulant factors leading to vascular dysfunction including vasoconstriction, vasospasm and vascular thrombosis.

D'Agnillo et al. (2000) reported that glutaraldehyde polymerized bovine Hb showed a slightly lower NO reactivity but had a higher auto-oxidation rate than

native bovine Hb. Interestingly, presence of $HbA_0$ or α-α crosslinked tetrameric Hb with bis(3,5-dibromosalicyl)fumarate (DBBF-Hb) reduced $H_2O_2$-induced bovine aortic endothelial cell apoptosis as measured by cell morphology and annexin-V binding assay (D'Agnillo 2000). Incubation of bovine arterial endothelial cells with Hb produced ferryl Hb after 3 h of hypoxia followed by 1 h of reoxygenation, a condition likely to be encountered in patient with traumatic or surgical hemorrhagic shock. Hb caused dose dependent reduction of intracellular glutathione (GSH) suggesting it elicited cellular oxidative stress. However, addition of anti-oxidants such as ascorbate, alpha-tocopherol, or trolox did not prevent Hb oxidation (McLeod 1999). In the presence of $H_2O_2$, DBBF-Hb and polymerized DBBF-Hb caused bovine aortic endothelial cell apoptosis which correlated with ferryl Hb formation (Goldman 1998). Rapid mixing of ferrous Hb with peroxynitrite (a highly toxic agent that forms from NO and peroxide) in vitro caused oxidation of ferrous irons and nitration of $\beta$ subunit tyrosine residues which led to Hb instability and heme loss (Alayash 1998). In another study, ferryl Hb formation and protein modification occurred during enzymatic peroxidation at an increasing order, polymerized bovine Hb > bovine Hb > bovine Hb-FMDA, a bovine $\beta$-$\beta$ crosslinked Hb with mono-(3,5-dibromosalicyl) fumarate (Alayash 1995).

If acellular HBOCs produce clinically significant amount of peroxynitrite formation in vivo, it can cause wide ranging damaging effects on cells, proteins and DNA due to its potent oxidant and nitrating properties. However, in hemorrhagic shocked swine, resuscitation with HBOC-201 did not increase evidence of oxidative potential. Hb-induced oxidative injury assessed by tissue 3-nitrotyrosine staining (a marker for peroxynitrite production) and tissue nitrosylation was similar in HBOC and hydroxyethyl starch treated control animals (Johnson 2006). Similarly, there was no increase in free radical generation following 30 mg/Kg of DCLHb infusion in rabbits subjected to 60 min renal ischemia and 10 min reperfusion (Pincemail 1995). Free radical production in this model was measured directly using EPR spectroscopy using alpha-phenyl N-tert-butyl-nitrone as a spin trap agent in the venous blood samples.

To date, however, most of Hb redox studies that reported toxic radical formation have been conducted in vitro under non-physiologic conditions. There is little data that positively confirm toxic oxy or nitrosyl radical formation in vivo in animals or humans following HBOC administration. Further studies are needed to clarify and validate the causative role of Hb redox reactions in the HBOC-mediated vasoactivity in vivo animal models and human subjects.

Of note, recently, HBOCs with anti-oxidant capabilities have been developed and showed some promising results against oxidant damages. Hb crosslinked with SOD and CAT (poly Hb-SOD-CAT) reduced heme mediated free radical generation and was protected from oxidant stress (D'Agnillo and Chang 1998). In addition, Hb conjugated with CAT and SOD using dicarboxymethylated PEG in 1:10 Hb/PEG ratio produced a large Hb-CAT-SOD (MW:1 mega Dalton) which were shown to have protective properties against severe free radical stress and reduced metHb formation (Nadithie and Bae 2010, 2011, 2012).

### *32.2.6 Other Putative Mechanisms*

Hemorphins are opioid peptides produced endogenously by peptic hydrolysis of beta globins of hemoglobin (Brantl 1986). One of these peptides Leu-Val-Val-hemorphin-7 (LVV-H7) was shown to elicit pressor effect and tachycardia in rats. Therefore, potential involvement of Leu-Val-Val-hemorphin-7 (LVV-H7) in the pressor effect of DCLHb was investigated in rats (Moisan 1998). The pressor effect of LVV-H7 was due to C-terminal amino acid sequence (HCOO-Arg-Phe) which activated sympathetic nervous system. However, the pressor activity of DCLHb was not altered by pretreatment with a LVV analog peptide that was known to inhibit the pressor effect of LVV-H7. Therefore, it was concluded that it is unlikely that the DCLHb mediated pressor effect in rats involves LVV-H7. In addition, Michel et al. (1996) proposed that carboxypeptidase M and N may also contribute to the HBOC mediated vasoactivity. They showed that carboxypeptidase M and N enzymatically removed the c-terminal arginine of unmodified Hb tetramer facilitating dissociation into dimers. However, Hb dimers will be quickly bound by the haptoglobin and removed from circulation and any excess over the haptoglobin binding capacity will be excreted through the urine. Besides, it was found that carboxypeptidase M and N were not effective in dissociating crosslinked Hb tetramers into dimers. Because most of current HBOC products in development are chemically modified Hbs, carboxypeptidase M and N would not have a significant role in HBOC mediated vasoactivity.

The HBOC-mediated vasoconstriction was also reported to occur via 20-hydroxyeicosa-tetraenoic acid (20-HETE) mediated mechanisms rat pial arterioles (Qin 2006). Following an exchange transfusion with zero-link bovine hemoglobin polymer, the diameters of pial arterioles were decreased by 20 % without altering arterial blood pressure. This constrictor response was attenuated by superfusing the surface of the brain with WIT-002 (10 μM), a 20-HETE antagonist, and was blocked by two chemically dissimilar selective inhibitors of 20-HETE synthesis, DDMS (*N*-methylsulfonyl-12, 12-dibromododec-11-enamide) and HET-0016 (*N*-hydroxy-*N*′-(4-butyl-2-methylphenyl)-formamidine). The pial arteriolar constrictor response to hemoglobin was not blocked by an inhibitor of nitric oxide (NO) synthase. Further, the inhibition of the constrictor response by DDMS was not altered by co-administration of the NO synthase inhibitor. It was concluded that the polymerized Hb mediated pial arteriole constriction was a physiologic homeostatic mechanism to regulate $O_2$ levels that is mediated by upregulation of 20-HETE production rather than by NO scavenging. These observations suggest that a primary mechanism for the HBOC vasoactivity may vary with blood vessel phenotypes.

Simoni et al. (2007) also reported that Hb gains angiotension converting enzyme (ACE) like activity when Hb is activated with $H_2O_2$. They proposed that ferrous Hb can serve as an angiotensin-1 receptor and its ferryl Hb form possess ACE-like activity. That is, Hb can convert angiotensin-1 to angiotension-2, 3, 4 and other potent vasopressor forms. They assert that this ACE-like property serves

as a possible mechanism contributing to the vasoconstrictive response following Hb administration. However, this proposition is based on observations from in vitro cell culture experiments and must be validated in more clinically relevant models of in vivo animal models and eventually in clinical studies.

## 32.3 Factors Affecting the HBOC-Mediated Vasoactivity

The degree of observed vasoconstriction and hemodynamic changes following HBOC treatment varied substantially with variety of factors including characteristics of a HBOC, experimental protocols, models used and other factors. Therefore, in this section, some key factors that contribute to the HBOC mediated vasoactivity will be briefly discussed.

### *32.3.1 Characteristics of HBOCs*

All current HBOC products contain an active $O_2$ carrying agent derived from human or animal Hb that is modified by chemical methods or via recombinant DNA technology. Consequently, HBOC products vary widely in molecular structure, MW and effective hydration volume and chemical/physical characteristics such as $O_2$ binding characteristics and solution properties. Some key characteristics of HBOCs that were shown to influence the vasoactivity will be discussed.

#### 32.3.1.1 Molecular Size/Effective Molecular Volume

It has been proposed that cell-free tetrameric Hbs (64 kD) and their dissociated dimmers can pass through the pores of the vascular endothelial fenestrations into the subendothelial space causing vasoconstriction (Bucci 2007). Vascular endothelial pores are reported to range from around 8 nm (small pores) to 50–60 nm in diameter (Rippe 1994). Therefore, in theory, HBOCs that are smaller than the pore size of endothelial fenestrations can extravasate into the subendothelial space where they can more easily scavenge endothelium derived NO diffusing across to the smooth muscle cells to effect GC-cGMP mediated relaxation.

To test the hypothesis, an unmodified tetrameric Hb and intramolecularly crosslinked Hb (sebacyl crosslinked bovine Hb, molecular diameter $\sim$6 nm) were administered to rats. While unmodified Hb quickly appeared in the urine and hilar lymph of the kidneys, sebacyl crosslinked Hb did not appear in the urine but extravsated into the hilar lymph of the kidneys (Matheson 2002). When this crosslinked tetrameric Hb was applied to the intraluminal side, NO scavenging mediated vasoactivity was observed in vascular beds with larger pores (e.g., splanchnic, renal) but not in vessels with tight endothelial junctions (e.g., cardiac,

cerebral) (Sampei 2005). This suggests that smaller MW components remain in HBOC products can pass through the endothelial fenestration to extravasate. In deed, an ultra high MW Hb prepared by zero-link chemistry (Zero-link bovine Hb, 20 mDa, molecular diameter of 50 nm) after 300 kD diafiltration, it did not appear in the hilar lymph (Matheson 2002). This product did not produce a significant rise in BP in anesthetized rats and conscious cats. In contrast to these findings, polymerization (to average MW 400 kDa) of αα intramolecularly crosslinked Hb with bis[3,5-dibromosalicyil] fumarate (DBBF-Hb) did not abolish but significantly attenuated pressor effect of DBBF-Hb when each was given 250 mg/Kg. Further, the attenuation was seen only when it was given as an isovolemic exchange but not as hypovolemic infusion (Abassi 1997). In a study of hemorrhagic shocked swine, reduction of low-MW Hb content ($\leq$64 kD) in HBOC-201 from 31 to 2 % significantly decreased vasoactive responses but further purification to 0.4 % did not eliminate vasoactivity (Rice 2008). The average molecular dimension of HBOC-201 is estimated to be around 25 nm. Therefore, this HBOC product used in the study still can pass through the larger pores of the endothelial fenestrations and cause vasoconstriction.

Sakai et al. (2000) reported that, in a conscious hamster dorsal skinfold model, HBOCs with lower MW or smaller molecular dimension produced greater BP elevation and vasoconstriction of resistance arteries. In this study, effects of 7 ml/Kg bolus hypervolemic infusion of four different HBOCs with molecular diameters of 7 nm (XLHb), 22 nm (PEG-Hb), 47 nm (HES-XLHb), 68 nm (polymerized XLHb), and 224 nm (PEG-HbV) were tested. Constriction of resistance arterioles was positively correlated with the level of hypertension and was inversely correlated with the diameters of test HBOC particles. Ideally, the HBOCs tested should have had the same characteristics except the molecular size/MW. However, for practical reasons, HBOCs prepared for this study also had different P50, COP and viscosity, and other characteristics. Therefore, it may be possible that the results obtained were influenced by other factors that also contribute to the HBOC vasoactivity. Of note, it should be noted that some of these parameters are not independently controllable.

In a more recent study, unanesthetized hamsters with a preinstalled window chamber were subjected to *hypervolemic* infusion of T-state polymerized bovine Hb prepared with different glutaraldehyde to Hb ratio (20:1–50:1). Following cumulative Hb doses to reach plasma Hb concentration of 0.5, 1.0 and 1.5 g/dl (Cabrales 2009), T-state polymerized bovine Hb of MW $<$ 500 kD elicited significant arterial BP elevation, vasoconstriction and decreased functional capillary density (FCD) at a dose dependent fashion and inversely related to crosslinking density (glutaraldehyde to Hb ratio). However, polymerized bovine Hb of MW $>$ 500 kD produced using a high crosslinking density (40–50:1) caused a moderate increase in arterial BP and depressed FCD only at the highest dose tested. In a subsequent study with a guinea pig 50 % blood volume exchange model, a bovine polymerized Hb (BPH) prepared by 30:1 glutaraldehyde to Hb ratio (MW $\sim$ 1.3 mDa) elicited the smallest mean arterial BP elevation (8.4 % increase over baseline) compared with those of BPHs prepared by 10:1 and 20:1

ratios (Baek 2012). Surprisingly, BPH prepared by 30:1 ratio had a longer intravascular retention time (T1/2 ~ 31 h) than that prepared by 40:1 ratio although it had a higher MW (~5 mDa) indicating faster clearance. These results indicate that HBOC MW or effective size in solution (e.g., hydration volume) is an important factor that influences on the HBOC vasoactivity. In theory, the larger MW or effective size of HBOC should have lower rate of extravasation thus elicit less vasoconstriction and BP elevation. However, it was suggested that an optimal MW range for HBOCs would be 500 kDa < MW < 2 mDa because it was found that a HBOC with MW > 5 mDa was cleared more rapidly possibly through the mononuclear phagocytic system (Baek 2012). Further studies are needed to confirm these findings in models that more closely resemble human cardiovascular physiology (e.g., pigs, nonhuman primates) and eventually in human subjects.

#### 32.3.1.2 Oxygen Affinity/$O_2$ Carrying Capacity

It is logical to assume that under normal physiologic conditions, $O_2$ supply in a specific vascular bed is regulated to match the target organ's metabolic needs. Therefore, it is reasonable to hypothesize that an $O_2$ capacity/$O_2$ affinity (P50) of a HBOC would affect vascular response because those properties along with blood flow determine how much $O_2$ would be available to the tissues. In this section, the role of P50 in the HBOC-mediated vasoactivity is reviewed.

In isoflurane-anesthetized rats (100 % oxygen), 50 % estimated blood volume (30 mL/kg) exchange transfusion with low oxygen affinity (P50 = 45 mm Hg) O-raffinose modified Hb (Hb raffimer) caused sustained increase in MAP, transient caudate tissue oxygen tension, and no change in regional cortical cerebral blood flow (Hare 2004). Interestingly, in 30 % hemorrhage shock-shed volume resuscitation rat model, Hb raffimer with either high (P50 = 11 mm Hg) or low $O_2$ affinity (P50 = 70 mm Hg) increased hippocampal oxygenation and had no significant differential effect on cerebral blood flow and tissue oxygenation (Hare 2006). In another study, effect of low an $O_2$ affinity (P50 = 70 mm Hg) hydroxyethyl starch conjugated Hb (HRC101, Hemosol) (Crawford 2007) on hippocampal oxygenation were tested in anesthetized mice following a near complete hemodilution (Hct ~ 1 %). In this extreme hemodilution model, hippocampal tissue oxygen tension was better maintained with lower $O_2$ affinity product (P50 = 70 mm Hg) than higher affinity one (P50 = 14 mm Hg) (Hare 2009).

In anesthetized cats, reduction of Hct by hemodilution with a non-oxygen carrying solution dilates pial arteriolar diameter and increases blood flow to maintain normal cerebral oxygenation (Koehler 2008). However, hemodilution with several different HBOC preparations with P50 ranging 4–34 mm Hg caused pial arteriolar vasoconstriction. It was concluded that the vasoconstriction occurred in response to decreased viscosity and increased plasma $O_2$ thereby triggering 20-HETE mediated autoregulatory vasoconstriction mechanism to maintain constant oxygen transport to brain (Koehler 2008).

Interestingly, in conscious hamsters, exchange transfusion with ultrahigh MW T-state polymerized bovine Hb (17 mDa) with low oxygen affinity (P50 = 40 mm Hg) elicited less BO elevation/vasoconstriction, better preserved FCD and tissue oxygenation than R-state polymerized bovine Hb with high oxygen affinity (P50 = 10 mm Hg) (Cabrales 2010). However, although both Hb preparations had the same Hb concentration (10 g/dl), the T-sate polymerized bovine Hb had a higher viscosity (11 vs 8 cP), lower MW (17 vs. 26 MDa) and lower COP (1 vs 7 mm Hg) than R-state polymerized bovine Hb. Therefore, interpretation of the results is confounding.

Sakai, et al. (1999) using hamster dorsal window model, studied effects of exchange transfusion (0–80 %) with HbV of varying oxygen affinity (P50 = 9–30 mm Hg) on the microcirculatory hemodynamics. They reported that HbV with P50 of 16 mm Hg might be the optimal value since there was the highest functional capillary density compared with those of HbVs with a lower (P50 = 30 mm Hg) or a higher (P50 = 9 mm Hg) $O_2$ affinity. However, there was substantial variations in arteriolar/venular diameters and blood flow values when blood exchange rate was 60 % or higher.

These results generally indicate that $O_2$ affinity of HBOCs appears to be a key factor contributing to the overall HBOC vasoactivity and blood flow with phenotypical differences in response among vascular beds. The results also raise a hypothesis that there may be different optimal P50 values for different HBOCs because they have different $O_2$ binding and delivery characteristics, $O_2$ carrying capacity. Further, even for the same HBOC, P50 may need to be optimized for each specific indication to closely match recipient's $O_2$ requirement (e.g., hemorrhagic shock, ischemic rescue, intraoperative hemodilution).

#### 32.3.1.3 Effect of NO Reactivity of HBOC

Because NO has a very high affinity for Hb, an oxygen carrier used in all HBOCs, it is generally believed that all HBOCs would scavenge endothelial NO and elicit vasoconstriction and BP elevation. Therefore, a reasonable hypothesis would be that level of HBOC vasoactivity would be positively correlated with its NO reactivity. Interestingly, however, Rolfs et al. (1998) reported that HBOCs with a higher NO binding affinity caused a milder or transient BP elevation than that with a lower NO affinity. Specifically, in rats with 50 % isovolemic exchange transfusion, HBOCs (αα-crosslinked Hb and Hb raffimer) showed an immediate and sustained BP elevations had the weakest NO binding affinity (Kd = 8 pm and 14 pM, respectively) while Hbs that showed either a transient (PHP) or no significant BP elevations exhibited the stronger NO binding (Kd = 4 and 5 pM, respectively). Based on the finding, they claimed that NO scavenging cannot be the primary mechanism for the acellular HBOC mediated pressor effect. In contrast, Olson et al. reported that the magnitude of BP elevations observed in animals are linearly correlated with in vitro NO oxidation or oxyhemoglobin NO dioxygenation rates (Doherty 1998; Olson 2004). Consistent with the findings, FDA recently

confirmed that all HBOCs it tested were vasoactive and hypertensive (Silverman 2009; FDA 2004). It would be a challenging goal to produce a Hb selectively modified to possess reduced NO reactivity but still maintains normal $O_2$ binding property. Nevertheless, using rational protein engineering and site specific mutagenesis methods, a new generation of recombinant HBOCs with acceptable vasoactivity and $O_2$ binding/delivery property is being developed (Varnado 2013).

#### 32.3.1.4 Effect of Solution Properties

(a) Colloidal osmotic pressure

Because HBOCs are oncotically active protein solutions, blood colloidal osmotic pressure (COP) may also change if HBOCs are intravenously administered resulting in fluid shift across the capillary wall. The net fluid shift is determined by the differences between the intra- and extra-capillary hydrostatic and COP values which is predicted by the Starling principle (Woodcock 2012).

Because most HBOCs have different solution properties from blood, repletion of lost blood volume with a HBOC could result in changes in blood solution properties notably in viscosity and colloidal osmotic pressure (COP) which affects hemodynamics and blood volume due to transvascular fluid shift. For example, a decrease in blood viscosity($\mu$) would increase blood flow (Q) by decreasing the vascular resistance assuming vascular radius (r), driving pressure ($\Delta P$) and vessel length (L) and other conditions remain the same. This can be seen in the simplified mathematical relationship first described by Hagen-Poisoulle equation:

$$Q = \left(\pi r^4 / 8\mu r\right)\Delta P$$

If HBOC administration results in hypo-oncotic, fluid will move out of the capillary into the interstitial space. Normally, excess interstitial fluid is promptly drained away by the lymphatic system but if the condition is prolonged it causes tissue edema increasing resistance to oxygen diffusion to the tissues. Conversely, hyperoncotic HBOCs could draw fluids into the vascular lumen increasing blood volume thereby could elevate BP. To prevent significant fluid shift, most HBOC products in development today are formulated as an iso-oncotic solution (25–28 mm Hg). However, some newer HBOCs are formulated as either hyper- or hypo-oncotic solution (Vandegriff 2003; Cabrales 2010; Harrington 2011) for which careful fluid management may be necessary. In many actual clinical situations, traumatic hemorrhage patients are often treated with large amounts of a crystalloid volume expander at the injury site because no matching blood or colloid alternatives are available. In those cases, volume overload with hypo-oncotic solution could cause significant tissue edema and associated problems.

(b) Viscosity

Hemodilution with a low viscosity HBOC could cause a significant reduction of blood viscosity leading to decreased shear stress mediated endothelial NO production, reflex vasoconstriction, decreased functional capillary density and impaired microcirculatory function (Intaglietta 1999). Based on this rationale, it was proposed that a HBOC with high viscosity would elicit less vasoconstriction via shear stress mediated mechanotransduction of endothelial NO release. Therefore, it would better preserve microcirculatory blood flow and tissue oxygenation (Intaglietta 1999). They asserted that a HBOC that will maintain near normal blood viscosity following administration would allow higher capillary perfusion pressure and maintain functional capillary density, a key parameter in tissue oxygenation and survival. Intaglietta and his collaborators have extensively tested this proposition by studying the effects of various low and high viscosity HBOCs and plasma expanders using a hamster dorsal skinfold window chamber model. Results of those studies indicated that a high viscosity, high $O_2$ affinity and low Hb concentration HBOC like PEG conjugated human Hb (MP4, Sangart) better preserves functional capillary density and $O_2$ delivery compared with other low viscosity HBOC products. Surprisingly, they found that certain high viscosity non-$O_2$ carrying plasma expanders such as PEG conjugated albumin solution were equally effective raising a question whether $O_2$ carrying property is necessary for a resuscitation fluid (Tsai 1994 2006; Caron 2000; Rochon 2004; Villela 2009). A more detailed discussion on this subject is provided in separate chapters of this book by Intaglietta and Acharya.

These results propose an intriguing new paradigm in the design of a resuscitation fluid that may better preserve microcirculation and tissue oxygenation but more studies are needed to validate the approach in more clinically relevant models.

### *32.3.2 Effect of Anesthesia*

One of the common side effects of general anesthesia is hypotension because most anesthetics cause vasodilation which lowers the BP. Spinal and epidural anesthesia (e.g., bupivacaine, chloroprocaine, lidocaine) could also cause hypotension due to sympathetic block which tends to cause peripheral vasodilation (Veering 2000). Young healthy people generally can tolerate a moderate hypotensive side effect but older and sicker patients are more susceptible to development of severe hypotension that could cause serious complications. If the hypotension is severe and prolonged, ischemic damages can occur to brain, heart and other critical organs (Finnerty 1954) especially in older and sicker patients with underlying cardiovascular pathologies as they have little or no myocardial functional reserve to reverse the hypotension. Therefore, if BP drops too low, a fluid and/or a vasopressor drug is typically administered.

Because of the hypotensive effects, anesthesia could mask or blunt the HBOC-mediated vasoconstriction and BP elevation. Indeed, a halogenated anesthetic was shown to attenuate DCLHb-induced contraction in porcine pulmonary vein rings while non-halogenated anesthetics isoflurane and fentanyl did not (Jing 1995). Propofol showed inhibitory effect only in low DCLHb concentration. However, epidural anesthesia (5 ml bupivacaine 0.25 %) had no effect on vasopressor effect of pyridoxylated human Hb-PEG conjugate (Apex Bioscience) in sheep (Bone 1999). This suggests that sympathetic block does not affect HBOC-mediated vasoactivity which is consistent with the observation that HBOC elicited vasoconstriction in cervical sectioned rats (Gulati and Rebello 1994a, b). This moderation in HBOC pressor response may be beneficial to certain patients such as those with existing hypertension. Lower BP would reduce blood loss in trauma and surgical patients from damaged blood vessels and reduce risk of vascular rupture due to severe hypertension. However, it could mask a more serious condition such as hypoperfusion of brain and other key organs that must promptly be treated. Therefore, when HBOC is administered to an anesthetized patient with risk factors mentioned above, patient's condition must be carefully monitored for any signs of serious clinical condition. Conversely, the hypertensive property of HBOCs could be beneficial in the prevention or modulation of anesthesia mediated hypotension. In fact, currently, there is an ongoing clinical trial to test efficacy of MP4 as an anti-hypotensive therapeutic for peri-operative hypotension under spinal anesthesia (Sangart clinical trials http://www.sangart.com/research/trials.htm, Olofsson 2011). It is being argued that the HBOC-mediated elevation of BP might actually be beneficial in hemorrhagic shock and other seriously hypotensive conditions because use of vasopressors were shown to improve survival in animal studies (Feinstein 2005; Sanui 2006). In fact, DCLHb was positively evaluated as a potential vasopressor in critically ill patients (Reah 1997).

### *32.3.3 Effects of Experimental Models and Protocols*

In pharmacologic studies, observed responses to the same drug may vary widely with experimental models and protocols used. Likewise, hemodynamic responses to HBOCs appear to vary substantially with test models (e.g., isolated organs n-vitro, in vivo animal models) and experimental protocols used (e.g., topload, isovolemic exchange transfusion/hemodilution, hemorrhagic shock-resuscitation). HBOCs have been tested in a variety of different preclinical test models including isolated organs/tissues and whole animal in vivo studies of mouse, rat, hamster, cat, rabbit, dog, pig, sheep and non-human primates (rhesus monkeys, baboons) subjected to various experimental models of hemodiluton and hemorrhagic shock-resuscitation. In addition, some advanced stage HBOCs were clinically tested in healthy human volunteers as well as selected patients with defined conditions (Silverman 2009; Jahr 2011; 2012).

It has been observed that there are species and vessel phenotype dependent variations in vascular responses to HBOC treatment (Robinson 1989; Vanhoutte 1984; Garcia-Villalon 1996). For instance, porcine pulmonary vessels are reported to be more sensitive to HBOCs than those of dogs and rats (Hart 1997; Freas 1995; Muldoon 1996). HBOCs elicit contraction without pre-stimulation with an agonist in porcine pulmonary arteries (Muldoon 1996), canine basilar arteries (Connor 1987), bovine coronary artery (Foneseca 2010) and human left internal mammary or radial arteries (Ritchie 2000) while aortic vessels from rats and dogs contract only after agonist induced precontraction (Kim 2005a, Hart 1997). Yet, DCLHb does not alter vascular tones of the isolated human umbilical artery or vein segments. In these vessels, L-NA, a NOS inhibitor, did not alter vascular tone either. These vessels were found to contain low basal cGMP levels (Jing 1996).

There appear to be variability in responses to the same agent even within the same animal/human subject, (Robinson 1989). For example, bovine pulmonary artery is more sensitive to HBOC treatment than coronary and portal vein (Foneseca 2010). Similarly, varying responses were reported in studies with isolated human vessels. DCLHb treatment does not alter vascular tone of isolated human umbilical arteries and veins (Jing 1996) while it significantly reduced carbachol and sodium nitroprusside (SNP) induced relaxation responses of isolated human left internal mammary and radial arteries (Vuylsteke 2001; Ritchie 2000). Interestingly, in these vessel types, DCLHb elicited contractions without agonist induced precontraction and presence of L-NAME had no effect. However, in isolated human internal thoracic arteries, Hb elicited a more predicted response: a dose-dependent reduction of Ach mediated relaxation following precontraction with phenylephrine (Golbasi 2003). In this vessel type, however, Hb did not significantly alter protamine-mediated relaxation, a putative endothelium independent vascular relaxant. In addition, L-NAME and methylene blue did not alter the protamine response either. Similar results were found in a study with isolated human radial artery; while DCLHb significantly reduced endothelium dependent relaxations, it did not alter the endothelium derived hyperpolarizing factor induced relaxations (Vuylsteke 2001). The vascular phenotypic differences in response to HBOCs suggest design of HBOCs with properties (e.g., oxygen affinity, vasoactivity, solution properties) specifically adjusted for an indicated clinical condition.

There are also significant variations in responses to a HBOC when different experimental protocols are used even within the same animal species. In a swine study of hemorrhagic shock with different severity (moderate controlled hemorrhage, severe controlled hemorrhage, severe uncontrolled hemorrhage and severe uncontrolled hemorrhage plus traumatic brain injury), HBOC-201 mediated vasoconstriction was significant for the first two infusions and inversely related to HS severity (Rice 2006). Interestingly, in another study of swine uncontrolled hemorrhage with or without TBI, HBOC-201 treatment did not cause significant reductions in renal or cerebral blood flow measured by color microspheres and Doppler flow probe indicating no vasoconstriction in those vessels (Malhotra 2003; Gurney 2004; Johnson 2006). In addition, HBOC treated animals showed consistently higher transcutaneous $PO_2$ without significant changes in lactic acid

and base deficit levels. These observations suggest that HBOC-mediated moderate vasoconstriction does not appear to impair critical organ blood flow and oxygenation. Further, treatment of trauma patients with a HBOC did not cause systemic and pulmonary hypertension (Johnson 1998).

Polymerization of DBBF-Hb significantly attenuated pressor effect of tetrameric DBBF-Hb but the attenuation was seen only when it was given as an isovolemic exchange but not as hypovolemic infusion (Abassi 1997) indicating how HBOC is administered influences the resultant vasoactivity.

It is not surprising to see reports of varying hemodynamic effects of HBOCs in clinical trials because, in most cases, different study protocols were used in different group of study subjects with disparate clinical conditions (Silverman 2009; Sloan 2010). Therefore, caution must be exercised not to draw blanket conclusions or when comparing results of multiple studies even the same HBOC product is used. Following two studies illustrate the case. In a study of patients undergoing elective abdominal aortic surgery, it was conclude that HBOC-201 administration at 3–9 ml/kg was of no clinical benefit compared with equivalent dose of hetastarch because HBOC-201 increased arterial pressure, systemic vascular resistance and depressed cardiac output resulting in lower $O_2$ delivery and consumption indices (Kasper 1996; 1998). Similar hemodynamic responses were also reported in a study with patients undergoing percutaneous coronary intervention procedures for coronary artery disease (Serruys 2008). Hypervolemic infusion of HBOC-201 at 15 or 30 g significantly increased systolic BP, pulmonary wedge pressure and systemic vascular resistance. Of note, however, no significant changes in coronary arterial diameter, blood flow and left ventricular stroke work were reported.

The inter-species and within species differences in vascular reactivity to Hb/HBOCs may stem from differences in basal cGMP levels and NO production. Indeed, there appears to be differential expression of NOS enzymes and/or soluble GC expressions across species and among the different vessel types in normal and pathologic conditions (Schermuly 2008; Bernardini 2005; Romero 2000; Fukuchi 1999).

Ideally, experimental models should closely simulate human clinical conditions (age, disease or injury) and experimental protocols that closely follow current standard medical practices. However, often selection of animal models is based on practical factors including availability in numbers, cost and ease of husbandry, etc. Therefore, small animal models like rodents and rabbits are more often used in preclinical studies that require a large number of animals. Therefore, it is important to understand limitations of each model especially regarding relevance of obtained data to actual human clinical situations (e.g., traumatic hemorrhage, surgical anemia, cardiac or limb ischemia). Many potential recipients of HBOC treatments are elderly patients with underlying co-morbidities including diabetes, hypertension and cardiovascular pathologies. Very few HBOC studies have been conducted in animal models with co-morbidities seen in human patients. Perhaps, this explains why preclinical studies failed to predict some of the serious adverse event observed in recent clinical trials. The selection of proper animal models is discussed in more detail in Chap. 26.

### *32.3.4 Effects of Co-Morbidities*

In recent HBOC clinical trials some serious adverse events (SAEs) were observed including severe systemic/pulmonary arterial hypertension, cardiac arrest and stroke (Silverman 2009).

Although vasoactivity and oxidative properties of HBOCs have been implicated (Natanson 2008; Alayash 2001c), no definitive causal relationship between HBOCs and the observed SAEs has been established. That is, in part, because the pathophysiologic response mechanisms involved in the study patients' clinical conditions (e.g., traumatic hemorrhage, cerebral or coronary ischemia) are extremely complex (Becker 2002; Levy et al. 2010; Cairns et al 2010; Chaudry 1992). Another confounding factor involved in HBOC clinical trials is the fact that many of the subjects included in the HBOC clinical trials were elderly with significant underlying comorbidities (e.g., hypertension, diabetes, cardiovascular diseases) in addition to the primary condition for which HBOC was indicated. A common feature of these co-morbid conditions is altered vascular structural integrity accompanied resulting in endothelial and/or smooth muscle dysfunction. In these patients, HBOC infusion may exacerbate the vascular injury/dysfunction, interfere with coagulation and host defense mechanisms by scavenging endothelium or immune cell derived NO as well as promoting release of proinflammatory mediators, oxy radicals and other cytotoxic agents.

The vascular endothelium has many important regulatory functions in health and disease: regulation of BP, blood flow, coagulation, blood volume and barrier function to pathogen invasion to blood stream. The vascular endothelium synthesizes/releases various regulatory mediators and signaling molecules including vasoactive agents (endothelium dependent relaxation factors, endothelium dependent constricting factors), coagulation factors, hormones, and inflammatory/stress factors (Stowe 1996). In addition, the endothelium expresses various receptors and mechano sensors that respond to changes in chemical stimuli/toxins in the blood, BP and blood flow (viscosity/shear stress). A ubiquitous mediator/messenger molecule involved in many of these processes is NO, a gaseous molecule synthesized and released by many tissues/cells including the vascular endothelium, immune cells and neuronal cells (Bentz 2000). Nitric oxide is now known to be involved in many normal and pathophysiologic processes including a crucial role in endothelial dysfunction in many disease states mentioned above. Damaged or dysfunctional endothelium would cause impaired NO synthesis/bioavailability along with coagulopathy and other functional abnormalities. Because of avid NO scavenging property, intravenous administration of HBOC may exacerbate such pathophysiologic conditions.

Under inflammatory co-morbid conditions (e.g., diabetes, atherosclerosis), the endothelium also releases primary cytokines that trigger release of a host of other mediators and effectors including adhesion molecules, matrix metalloproteinases and reactive oxygen species. In parallel, these primary cytokines induce the expression of the messenger cytokine IL-6, particularly in smooth muscle cells. IL-6

then travels to the liver, where it elicits the acute-phase response, resulting in the release of C-reactive protein, fibrinogen, and plasminogen activator inhibitor-1. All these inflammatory markers and mediators, released at different stages in the pathobiology (Packard 2008). In these conditions, structural and functional integrity of the endothelium are compromised. In addition, genetic predisposition and certain acquired habits/lifestyle (e.g., smoking, alcoholism, drug abuse) could also promote endothelial damage and dysfunction. These conditions could cause acute or chronic endothelial dysfunction. In addition, damaged endothelium promotes platelet (PLT) activation and aggregation. At the same time, the activated PLTs release procoagulation factors that promote vascular thrombosis. Damaged blood vessels also attract activated phagocytic leukocytes that release $H_2O_2$, reactive oxygen species (ROS) and other cytotoxic agents as a part of host defense mechanisms. This initial host defense mechanism causes further vascular inflammation and compromise endothelial integrity and function. This in turn could lead to inadequate blood flow and oxygen delivery to critical organs leading to organ dysfunction and failure.

Under such conditions, presence of Hb/HBOC will further disrupt endothelial function as it scavenge NO and promote oxy- and nitrosyl radicals that could further exacerbate endothelial dysfunction and vascular dystonia.

This may be one of the reasons why preclinical animal studies failed to predict AEs revealed in human HBOC clinical trials because most animal studies were conducted using healthy young animals that do not simulate elderly human subjects with various co-morbidities. However, some recent reports indicate that HBOC administration attenuates IL-8 gene expression and post-injury hyperinflammatory responses in injured patients (Johnson 2003; Sheppard 2004). In addition in human lung microvascular endothelial cell and macrophage culture experiments, polymerized Hb treatment attenuated LPS-induced cytokine and intercellular adhesion molecule-1 protein cell surface expression by induction of anti-inflammatory cytoprotective protein HO-1 (Cheng 2005; Roach 2009).

In support of this hypothesis, a recent study (Yu 2010) reported that polymerized pyridoxylated human Hb product (PolyHeme®, Northfield Labs) elicited hypertension in diabetic mice (known to have endothelial dysfunction) but not in normal mice. This finding supports the hypothesis that pre-existing endothelial pathologies could potentiate HBOC-mediated vasoconstriction. This has significant clinical implications because older sicker subjects/patients that have underlying cardiovascular pathologies (hypertension, diabetes, atherosclerosis) with endothelial dysfunction are more likely to develop clinical conditions that will require blood or HBOC transfusion.

Recently, endocytosis of some HBOCs (Dex-BTC-Hb, a 300 kDa HBOC product) by guinea pig aortic endothelial cells has been reported (Smani 2005; 2006; Faivre-Fiorina 1999). A recent discovery of Hb scavenger receptors (CD163) in the vascular endothelial cells further supports this possibility (Schaer et al. 2006). If, indeed, HBOCs are taken up by the endothelial cells through a receptor mediated pathway, it is plausible that the endocytosed HBOC and some of its components may interact with cytoplasmic (e.g., NOS) and mitochondrial

enzymes that could also lead to abnormal vascular response further confounding the mechanisms involved.

Furthermore, as indicated above, traumatic hemorrhage also activates coagulation and immune mechanisms that could directly and indirectly affect vascular tone and BP. Blood vessels partially or totally obstructed by injuries or blood clot would cause upstream BP elevation. In addition, local or systemic inflammation or tissue edema could also aggravate vasoconstriction. HBOCs could interfere with these mechanisms leading to higher incidences and/or worsened adverse effects.

Of note, it is well known that trauma and hemorrhage elicit immunosuppression due to damaged natural barriers and lost immune cells and other protective factors during hemorrhage. Thus, traumatic hemorrhage victims more susceptible to subsequent infections and sepsis that contributes to the high mortality (Chaudry 1992). The exact mechanism by which the immunosuppression occurs has not been fully elucidated. Obviously, reduction in leukocytes and other immune factors lost during hemorrhage is a factor. In addition, suboptimal oxygen supply and nutrients to the key immune organs due to compromise circulation and ischemic conditions. In distributive shock (e.g., septic shock), inducible NOS is activated producing excessive amount of NO leading to refractive hypotension. In those cases, treatment with NO scavenger such as acellular Hb or HBOCs may be helpful in restoring BP. Indeed, in animal studies, administration of Hb better preserved BP especially when combined with NOS inhibitors (Kim 2001b, 2002). However, in a recent clinical trial, PEGylated human Hb in patients with distributed shock did not show significant clinical benefit (Kinasewitz et al. 2008). Of note, both acellular and cellular type HBOCs are also cleared by the monophagocytic system (MPS), a principal initial defense against pathogen invasion. (Hietbrink 2006). Therefore, infusion of large amounts of these HBOCs could overwhelm the MPS potentially compromising pathogen clearance. Indeed, it was found that there is a temporal relationship between moratlity and Hb infusion in septic animals (Kim 2004c). Therefore, in patients with developed sepsis or in subjects who are likely to develop sepsis (e.g., trauma patients with open wounds), HBOC administration may lead to increased incidences of severe adverse events and death. Clearly, more studies are needed to investigate this important question.

## 32.4 Summary and Conclusion

Most, if not all, current acellular Hb-based oxygen carriers elicit hypertensive effect when intravenously administered to animals and humans because they cause vasoconstriction. The mechanism(s) of this pressor effect of HBOCs have not been definitively elucidated. Hb scavenging of endothelial NO appears to play a major role in the HBOC-mediated pressor responses but ET-1 and other mechanisms may also contribute to the overall outcome depending on the patient's condition and a specific HBOC product used.

Regarding the mechanisms for the HBOC mediated BP elevations and vasoconstrictions, it is possible that one or more mechanisms may work concomitantly or in concert to effect the overall vasoactivity depending on specific clinical conditions. Treatment with HBOCs has recently been implicated to increase risk of myocardial infarction and other SAEs without substantiated data to support the claim. Higher incidences of certain adverse events in HBOC clinical trials may be in part due to imbalance in comorbidities of subjects in the study and control groups. Pathophysiology of trauma and hemorrhage alone are already exceedingly complex; HBOC administration to patients with multiple co-morbidities makes it even more complex and challenging. More studies are needed to investigate pathophysiologic responses to HBOCs resuscitation especially when significant cardiovascular comorbidities are present.

Some HBOC products claimed that their products do not elicit hypertensive effects. However, according to a recent FDA report (Silverman 2009), "all current HBOC products in or previously in development are vasoactive at the doses proposed for resuscitation or for blood replacement".

One of the obstacles to more effective studying HBOC-mediated BP elevation/pressor effect in clinical trials is insufficient publicly available information regarding detailed characteristics of HBOC product used, notable medical conditions of the study patients (e.g., existing conditions) and nature/clinical course of AEs/SAEs, treatments provided for the AEs and other significant clinical events (co-medications).

Based on the review of published data, severity of vasoconstriction (BP elevations) responses following intravenous HBOC administration varies substantially with variety of factors including product characteristics ([Hb], MW, P50, viscosity, etc.), dose and rate of administration, animal models protocol/protocols used. Generally, topload or exchange transfusion protocols show more notable BP elevations than hemorrhagic shock-resuscitation model. In addition, the degree of BP response was also varied with animal species; pigs were more sensitive than rats and dogs. Vascular response to HBOC varied also with vessel phenotypes (e.g., aorta, coronary artery, pulmonary artery/vein, portal vein). In addition, underlying co-morbidities (e.g., hypertension, diabetes, cardiovascular disease, inflammation, etc.) that are known to accompany pathologic changes in vascular structure and function could affect overall vascular response to HBOC administration. In addition, certain anesthetics may blunt or mask HBOC-mediated BP elevation and vasoconstriction. Finally, patients are often medicated with vasoactive drugs (e.g., vasopressors for shock, nitrovasodilators for cardiac procedures, Ca channel blockers/ACE inhibitors for hypertension) prior to or concomitantly with HBOC administration (co-medication) which could mask or alter hemodynamic responses to HBOC treatment. All of these variables and factors that influence the overall response to HBOC made interpretation difficult and complicated efforts to elucidate the mechanism(s) involved in HBOC-mediated vasoactivity and their causal relationships to the observed AEs in HBOC clinical trials. Unless we have reasonable answers to these questions, it would be

extraordinarily challenging to develop a newer generation of HBOCs that are without vasoactivity and other adverse effects.

As the 'baby boomer' generation age, more people will need medical procedures that require blood transfusion while donor pool of younger population shrinks straining already tight global supply of safe blood (WHO 2011). Therefore, aside from military trauma, there will be a strong civilian demand for an alternative to allogeneic blood transfusion in the coming decades. Therefore, it is highly desirable that a new generation of safer and more effective HBOC products with acceptable vasoactivity be available in the very near future.

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# Chapter 33
# HBOCs and Cardiac Integrity

**T. N. Estep**

## 33.1 Introduction

Hemoglobin (Hb) based oxygen carriers (HBOCs) have yet to be approved for human use in most countries due to adverse events observed in clinical trials. In a widely publicized meta-analysis of sixteen clinical studies utilizing five different HBOCs, Natanson and colleagues concluded that there was a significantly increased risk of myocardial infarction (MI) in treated patients (Natanson et al. 2008). While this analysis and its conclusions have been challenged on a number of methodological and scientific bases (Greenburg and Pittman 2013), the fact that myocardial lesions have been observed after HBOC treatment in some preclinical studies has reinforced this concern (Silverman and Weiskopf 2009; Burhop et al. 2004). On the other hand, there is an extensive literature demonstrating that HBOC administration supports and preserves myocardial function. This chapter summarizes research pertaining to the effects of HBOCs on cardiac function and cell viability.

## 33.2 Variables of HBOC Formulation and Use Affecting Cardiac Integrity

A number of properties of HBOC formulations influence the functionality and cellular integrity of heart tissue (Table 33.1). In addition, specific details of HBOC infusion protocols can impact hemodynamics (Table 33.2). These variables are discussed in the context of proposed mechanisms of action.

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**Table 33.1** Properties of HBOC formulations that may affect cardiac integrity

| Property or variable | Possible influence on cardiac performance and cellular integrity |
|---|---|
| Physical/chemical characteristics of hemoglobin active principal | |
| Molecular size and size distribution | -Rate of extravasation into heart tissue |
| | -Alteration of vasoactive response |
| | -Alteration of generation of cardiac lesions |
| Oxygen binding affinity | -Ease of direct oxygenation of heart |
| | -Indirect autoregulatory responses to oxygenation of tissues |
| Rate of interaction with nitric oxide | -Degree of HBOC vasoactivity |
| | -Alteration of internal heart metabolism and cell signaling |
| | -Platelet activation |
| Generation of reactive oxygen species | -Generation of reactive species damaging to cellular integrity |
| | -Interaction of ROS with NO signaling pathways |
| Rate of nitrite reduction to nitric oxide | -Mitigation of effects of NO scavenging |
| Viscosity | -Direct hemodynamic influence on heart performance |
| | -Indirect responses to changes in tissue perfusion |
| Oncotic pressure | -Intravascular fluid volume expansion and resulting effects on hemodynamics |
| Modification chemistry | -Hb modifying agents may exhibit biologic activities in their own right |
| | -Metabolic breakdown products of agents may not be inert |
| HBOC formulation | |
| Hemoglobin concentration | -Solution viscosity and oncotic pressure |
| | -Affects amount of concurrent fluid volume administered along with active principal |
| Vehicle composition | -Inclusion of vasoactive excipients can affect hemodynamic response |
| | -Inclusion of antioxidants could affect generation of ROS |
| | -Other activities of vehicle excipients may be manifested in heart |
| pH | -pH and buffering capacity of HBOC may influence blood pH |
| Purity | -Residual unmodified Hb or tetramers may influence size dependent parameters |
| | -Residual phospholipids, non Hb proteins, modification agents, or endotoxin can exhibit toxic side effects |

**Table 33.2** Aspects of HBOC infusion protocols that may affect cardiac integrity

| Property or variable | Possible influence on cardiac performance and cellular integrity |
|---|---|
| Anesthesia | Anesthetics can mitigate vasoactivity and alter normal hemodynamic responses |
| Concomitant medications | -Antihypertensives or antioxidants can ameliorate side effects<br>-Interactions of HBOCs with many commonly used medications are unknown |
| Additional fluids | Intravenous fluids infused before, during or after HBOC solutions can affect hemodynamic responses and cardiac loading |
| Posology | -Dose, rate and timing of HBOC solution infusion can alter hemodynamic responses<br>-Different responses may be observed with volume load versus exchange transfusion versus resuscitation protocols |
| Preconditioning | Previous exposure to ischemia or HBOC infusion can alter vasoactive response and development of cardiac reperfusion injury |
| Comorbidity | Ongoing disease may predispose to injury or dysfunction |

### *33.2.1 HBOC Properties Directly Affecting Hemodynamics*

The most direct effect of HBOCs on cardiac function is the ability to transport oxygen, which is primarily determined by hemoglobin concentration and the oxygen binding function; the latter is typically characterized by $P_{50}$, oxygen partial pressure at which Hb is half saturated, and n, the Hill parameter, which denotes the degree of cooperativity of oxygen binding (Winslow 1992). Given the fact that oxygen extraction in the left ventricle of the heart is 70–75 % even under resting conditions, the primary mechanism by which heart muscle can compensate for anemia or increased oxygen binding affinity is by increasing coronary blood flow (Dunker and Bache 2008; Woodson and Auerbach 1982). While normal mammals can substantially increase this flow in compensation, this may become difficult in compromised patients (Dunker and Bache 2008).

Blood hemodynamics are also affected by the viscosity and colloid osmotic pressure (oncotic pressure) of HBOC solutions, especially when they are infused in high volumes. As blood viscosity decreases, cardiac output (CO) tends to increase (Spahn et al. 1994). In addition, viscosity also influences tissue perfusion which in turn may alter the systemic vascular resistance (SVR) against which the heart must pump (Intaglietta 1999). Oncotic pressure is a major determinant of the degree to which fluid is absorbed into the bloodstream (Guyton 1992), and it has been demonstrated that highly oncotic HBOC solutions are quite effective at expanding blood volume (Posner et al. 2003; Fischer et al. 1999).

### *33.2.2 HBOC Vasoactivity*

HBOC effects on the heart are intimately related to the property of vasoactivity which is discussed at length elsewhere in this volume (Kim 2013) and summarized here to enable an appropriate discussion of cardiac effects. This vasoactivity is manifested in vitro as contraction of isolated vessels exposed to Hb (Freas et al. 1995) or in vivo as an increase in mean arterial pressure (MAP) and/or the resistance of one or more vascular beds after intravenous infusion (Sharma et al. 1994; Malcom et al. 1994). A primary mechanism for this response is the extravasation of molecular Hb into the interstitial space between the vascular endothelium, where nitric oxide (NO) is synthesized, and the smooth muscle cells, which control vascular wall tension and where NO induces muscle cell relaxation (Freas et al. 1995; Nakai et al. 1998; Olson et al. 2004; Kelm and Schrader 1990). The extravasated Hb then reduces the interstitial concentration of NO which avidly binds to deoxyhemoglobin and reacts with oxygenated hemoglobin to yield methemoglobin and nitrate (collectively known as NO scavenging) (Olson et al. 2004; Kelm and Schrader 1990; Sharma et al. 1987; Eich et al. 1996). This in turn results in smooth muscle contraction and an increase in vascular resistance. This hypothesis predicts that vasoactivity should be reduced by increasing the molecular size of Hb molecules to inhibit the rate of extravasation, and/or modifying hemoglobin molecules either chemically or genetically to reduce the inherent rate of NO scavenging. Both of these predictions have been supported by experimentation (Nakai et al. 1998; Doherty et al. 1998; Matheson et al. 2002; Sakai et al. 2000; Sampei et al. 2005). In addition, results suggest that other pathways, such as those involving endothelin (Schultz et al. 1993), prostaglandins (Qin et al. 2006), sensitization of α-adrenocepters (Gulati and Rebello 1994), or autoregulatory responses to the act of oxygen delivery (Tsai et al. 2003), may be involved as well. These pathways are not mutually exclusive and are known to interact. Notably, vasoactive responses vary from species to species, organ to organ, and even among different vessels within the same organ due to differences in the degree to which various vessels utilize NO to regulate homeostasis and the permeability of different vascular beds to HBOCs (Dunker and Bache 2008; Freas et al. 1995; Sampei et al. 2005; Wellum et al. 1980). One important implication of these facts is that vasoconstriction is not uniform throughout the vasculature after HBOC infusion, resulting in a redistribution of blood flow. In rats, dogs and swine there is a preferential redistribution of flow to the coronary circulation (Sharma et al. 1994; Mongan et al. 2009; Gulati et al. 1994; Gulati and Sen 1998; Kingma et al. 2002). Species differences are also important with respect to extrapolation of experimental results to humans. For example, it is known that coronary flow in dogs is unchanged after the infusion of NO inhibitors, that of swine and humans is modestly decreased, while in isolated rodent hearts there is a marked reduction (Dunker and Bache 2008), reinforcing the notion that results in swine are more predictive of those to be expected in humans (Muir et al. 2011). This also explains why more coronary vasoconstriction is seen in isolated rodent heart preparations (MacDonald et al. 1990; MacDonald and Winslow 1992) than in vivo with swine and humans (see below).

### 33.2.3 HBOCs and the Nitroso-Redox Balance in the Cardiovascular System

In addition to affecting vascular tone, NO and its derivative reactive nitrogen species (RNS) are known to perform important cell signaling functions in the heart at low concentrations, but may become cytotoxic at higher levels (Zimmet and Hare 2006; Ziolo et al. 2008). NO and RNS can inhibit mitochondrial respiration and both induce or inhibit cell death by a variety of mechanisms (Brown and Borutaite 2002, 2007). Some of these seemingly contradictory results are a consequence of the fact that at least three different nitric oxide synthase (NOS) enzymes are found in different subcellular locations within heart tissue, and these enzymes can act independently and in opposition on heart structure and function (Ziolo et al. 2008; Barouch et al. 2002). Perhaps not surprisingly, NO and RNS have been implicated as both mediators and inhibitors of reperfusion injury in the heart (Wang and Zweier 1996; Bolli 2001). In somewhat analogous fashion, reactive oxygen species (ROS) also perform both regulatory and pathologic functions in the heart in which red cell hemoglobin and cardiac myoglobin are known to participate (Zimmet and Hare 2006). For example, both hemoglobin and the myoglobin generate the ROS superoxide when either protein is oxidized, and both superoxide, oxidized globin proteins and hemin released from degraded globin proteins may participate in a variety of reactions to generate additional ROS species (Zimmet and Hare 2006; Everse and Hsia 1997; Schaer et al. 2013). Furthermore, the RNS and ROS pathways interact at multiple points (Zimmet and Hare 2006). Finally, it has recently been recognized that hemoglobins possess a nitrite reductase activity which results in the production of NO from nitrite that may counteract some of the consequences of NO scavenging (Gladwin and Kim-Shapiro 2008; Lui and Kluger 2010; Rodriguez et al. 2009). Some HBOC chemical modifications increase the rate of this reaction (Lui et al. 2008). Thus, HBOCs can generate a variety of ROS and RNS which may be toxic in excess. On the other hand, the cardiovascular system, as well as the body as a whole, contain a number of enzymatic and non-enzymatic antioxidants that mitigate the effects of nitrosative and redox stress (Everse and Hsia 1997; Schaer et al. 2013; Giordano 2005).

### 33.2.4 HBOC Formulation Variables

Hb concentration in an HBOC formulation affects the viscosity, oncotic pressure, and dosing schedule of the resulting product. In addition, Hb concentration influences the total volume of fluid which patients receive which may be important in high dose indications. Likewise, the pH of the formulation can have measurable impact on blood pH, as Hb has some buffering capacity within the physiologic range. This may be relevant in situations where correction of acidosis is one of the

desired outcomes. Other vehicle constituents can have biologic activities of their own. For example, some HBOC preparations contain antioxidants which may participate in the oxidation/reduction reactions mentioned above (Dubé et al. 2008). Lactate is a frequent component that has aroused concern due to potential adverse metabolic effects of the d isomer (Valeri et al. 2006). A few HBOC formulations have included acetate which has been associated with myocardial depression and hemodynamic instability in intensive care unit patients (Vincent et al. 1982; Leunissen et al. 1986). Finally, although great strides have been made in ridding HBOC preparations of toxic residuals, some published studies utilized material that was contaminated with endotoxin. Endotoxin is known to cause cardiac dysfunction which is mediated through cytokines that alter the myocyte nitroso-redox balance (Chagnon et al. 2006; Parker 1998; Berkowitz 2007). Furthermore, endotoxin toxicity is increased in the presence of Hb (Su et al. 1997).

### *33.2.5 Protocol Variables*

Hemodynamic responses to HBOC administration are dose dependent, at least over some dose ranges (Malcom et al. 1994; Gulati and Sen 1998). In addition, different anesthetics can affect the hemodynamic response to HBOCs, which may in part explain conflicting results in the literature (Muir et al. 2011). For example, porcine pulmonary vein contraction elicited by diaspirin crosslinked hemoglobin (DCLHb) was shown to be diminished by halothane, but not isofluorane (Jing et al. 1995). Halothane anesthesia was also shown to diminish the increase in MAP and pulmonary artery pressure (PAP) observed after infusion of pyridoxylated hemoglobin polyethylene conjugate (PHP) into sheep (Bone et al. 1999). Likewise, hemodynamic responses to HBOCs can be modulated by antihypertensives, preceding or concurrent fluid therapy, and the rate and route of HBOC administration (Ning et al. 2000; Lee et al. 2002), and, as noted above, hemodynamic responses are also species dependent (Dunker and Bache 2008; Wellum et al. 1980). Thus, comparisons between studies and implications for human cardiac toxicity must take into account a number of protocol variables.

## 33.3 Preclinical Data

Due to the many parameters and biochemical reactions that may affect the interaction of HBOCs with heart tissue, in vivo evaluations are necessary to assess the overall impact. Such studies have been conducted since the early 1970's, but many early preparations were impure and poorly characterized. Also, it is generally agreed that formulations of unmodified hemoglobin are unlikely to serve as useful products because of renal toxicity, high oxygen affinity, and short intravascular

persistence. This discussion will therefore focus on studies performed with modified and highly purified HBOC formulations.

### 33.3.1 Volume Load and Moderate Blood Exchange Studies

HBOC effects on cardiac performance have been assessed in several normal animal models. Bovine glutaraldehyde polymerized hemoglobin (HBOC-301) infusion into rats resulted in an increase in MAP and a decrease in cardiac index (CI, CO divided by animal surface area), heart stroke volume (SV), and heart rate (HR) (Irwin et al. 2008). When DCLHb was infused into rats there was an increase in MAP and systemic vascular resistance (SVR), while HR, CO and SV were not significantly changed (Sharma et al. 1994). Blood flow to most organs was not significantly altered, but flow to the heart increased approximately three-fold. The fraction of CO going to the musculoskeletal system was significantly decreased. When HBOC-301 was infused into conscious dogs, MAP was elevated by 43 % and coronary blood flow increased 93 % (Loke et al. 2000). In this study the maximum initial velocity of left ventricle contraction ($dP/dt_{max}$) was not significantly affected, but myocardial oxygen consumption doubled and cardiac metabolism shifted from the use of free fatty acids to lactate and glucose. When this HBOC was infused into anesthetized dogs, CO, HR, and $dP/dt_{max}$ were decreased and MAP, SVR, and left ventricular end-diastolic pressure (LVEDP) were increased (Muir 3rd et al. 2000). The authors concluded that the increased SVR was likely responsible for the decreased CO, and that increases in SVR and blood volume contributed to the LVEDP increase. Stepwise exchange transfusion of up to 50 % of blood volume with a more highly purified version of this HBOC (HBOC-201) into lightly anesthetized swine resulted in modest increases in MAP, pulmonary artery pressure (PAP), and SVR, but no significant changes in CI or global oxygen consumption (Mongan et al. 2009). In addition, there was no change in regional blood flow to the heart or seven other organs after HBOC-201 transfusion, with the only change being a decrease in flow to skeletal muscle. In a similar protocol, Muir et al. also found that oxygenation of heart tissue was increased after HBOC-201 infusion (Muir et al. 2011).

These preclinical data are consistent in that HBOC administration usually results in an increase in MAP and SVR which is sometimes associated with a decrease in CO. Nevertheless, blood flow to the heart is maintained or even increased and cardiac function is well preserved.

### 33.3.2 High Blood Volume Exchange

In anesthetized swine, Meisner et al. compared the response to DCLHb with that of an oncotically matched human serum albumin (HSA) solution during stepwise

isovolemic hemodilution to a hematocrit of 1 % or until myocardial ischemia became evident (Meisner et al. 2001). With HSA infused animals, ischemia was manifested at a hematocrit of 6.1 %, with five of the six pigs exhibiting ST-segment depression during electrocardiogram (ECG) analysis. DCLHb treated animals showed no evidence of myocardial ischemia at a hematocrit of 1.2 %. This is not surprising in light of the fact that the arterial oxygen content ($Cao_2$) and whole body oxygen delivery index ($Do_2I$) were twice as high in the DCLHb compared to HSA infused animals at their respective limits due to the higher total hemoglobin concentration in the former. MAP, SVR, and PAP were elevated during the initial exchange transfusions with DCLHb, but at extreme hemodilution only the PAP remained elevated above baseline values. CI remained unchanged throughout the protocol and blood flow to the heart and left ventricular contractility were maintained in the animals receiving the HBOC. Perfusion and oxygenation of skeletal muscle was decreased in both groups of animals.

Purified, glutaraldehyde polymerized bovine hemoglobin solutions have been exchange transfused at high volume into rats, dogs and sheep. In conscious rats exchange transfused to a hematocrit of less than 3 %, CI and oxygen delivery were well maintained during the subsequent 4 h observation period, although MAP and SVR increased by 30 % (Waschke et al. 1993). Standl and coworkers reported two studies in anesthetized dogs which were hemodiluted to target hematocrits with nonoxygen transporting solutions and then further exchange transfused with HBOC or comparator formulations. In the first study foxhounds were hemodiluted to a hematocrit of 10 % with a 6 % hetastarch solution (HES) and then received stepwise infusions of either stored dog red blood cells, fresh dog RBCs or HBOC solution until the total blood hemoglobin levels were increased by 1, 2 or 3 g/dl (Standl et al. 1996). As expected, all dogs responded to the initial HES hemodilution by increasing HR and CO, while decreasing SVR. Hemodynamic changes tended to be reversed after infusion of all three Hb containing formulations, but oxygen delivery ($DO_2$) was less with the HBOC and oxygen extraction was greater. In particular, CO declined in a similar fashion and MAP and SVR were increased to a similar extent with all three Hb formulations. During the Hb reinfusion phase of this study, skeletal muscle oxygen tension was restored more quickly with the HBOC infusion. In a second study Beagles were hemodiluted to hematocrits of 20 % with lactated Ringer's solution (LR) and then further exchanged transfused to hematocrits of 15, 10 and 5 % with either HES solution or HBOC (Standl et al. 1997). HR, CO and blood flow progressively increased in the HES transfused dogs until they reached a hematocrit of 5 % at which point they were euthanized because of cardiopulmonary decompensation. HR, CO and blood flow decreased after the initial HBOC infusion and remained lower than baseline upon additional infusions. However, HBOC treated dogs exhibited good hemodynamic stability at a final hematocrit of 2 % and skeletal muscle oxygen tension was significantly higher than HES infused animals. Furthermore, arterial lactate levels were significantly lower in the HBOC group. MAP was similar and comparable to baseline values in both groups throughout the procedure, but some vasoconstriction was manifested in the HBOC treated animals as an increase in SVR.

In an exchange transfusion study with splenectomized, conscious sheep, Lee and coworkers first hemodiluted animals to a hematocrit of 20 % with LR, followed by stepwise exchange transfusion of HBOC solution for blood until hematocrits were less than 4 % (Lee et al. 1995). During the HBOC exchange, there was a significant increase in MAP and PAP, but HR and CO remained relatively stable and oxygen consumption was maintained at near baseline level even at a final hematocrit of 3.2 %. As was observed with dogs, oxygen delivery was decreased in the HBOC treated animals, probably due to a decrease in the total hemoglobin concentration, but oxygen consumption was maintained by a higher degree of oxygen extraction. All HBOC animals tolerated the exchange well and exhibited no signs of distress while breathing room air, even at the final hematocrit of 3.2 %. These animals also survived long term. In contrast, control animals exchange transfused with HES were unable to survive the initial exchange transfusion protocol.

A similar pattern of maintenance of oxygen flux in the face of increased MAP and SVR was observed when Hb Raffimer, a human hemoglobin crosslinked with oxidized trisaccharide *o*-raffinose, was exchange transfused into anesthetized rats to effect a 50 % blood volume replacement (Filho et al. 2005). In this experiment the authors concluded that HBOC infused animals exhibited better cardiac performance than control animals infused with blood as evidenced by maintenance of the CI in the former group compared to a significant decrease in the latter. When high volumes of the two PEG derivatized human hemoglobins, PHP and MP4, a polyethylene glycol derivatized human hemoglobin formulation, were exchange transfused into anesthetized swine or conscious hamsters, respectively, CI increased (Vaslef et al. 2001; Cabrales et al. 2005). With the former this increase was accompanied by an increase in MAP and PAP, while in the latter case MAP decreased, implying a decrease in SVR. This difference in response could be due to the differing oxygen binding affinities of the two preparations ($P_{50}$ of 24 mm Hg for PHP, versus 5.4 mm Hg for MP4), differences in formulation details, species differences, or the use of anesthetized versus conscious animals. Both of these PEG derivatized Hbs have a higher oncotic pressure than other human Hb preparations on a per gram basis which probably enhances their ability to perfuse tissue through volume expansion and thereby maintain CO (Yabuki et al. 1990; Vandegriff et al. 1997).

These data demonstrate that a variety of HBOC solutions can support cardiac function at otherwise lethal hematocrits during isovolemic exchange transfusion, even when vasoconstriction is evident.

### *33.3.3 Resuscitation*

Given the potential use of HBOCs in resuscitation from hemorrhagic shock, extensive preclinical testing has been performed in animal models of this indication. When 10 ml/kg of a 7 g/dl DCLHb solution was exchange transfused into conscious pigs in an isovolemic fashion after a 35 ml/kg hemorrhage, MAP was

restored to baseline levels by the end of the exchange and increased further during a subsequent volume replacement using lactated Ringer's (LR) solution (Dunlap et al. 1995). This protocol was designed to simulate a severe hemorrhagic insult, followed by an initial resuscitation when blood loss was continuing and then volume replacement after control of hemorrhage. Comparator groups included resuscitation with human serum albumin (HSA) followed by LR or more HSA and a group resuscitated and volume replaced with LR only. Survival in the LR:LR group was poor. MAP in the two HSA groups remained below that of the DCLHb groups throughout. CI increased upon resuscitation in all groups, but more so in animals receiving the HSA. SVR was significantly higher, and HR lower, in animals infused with DCLHb. Base excess was restored to baseline more rapidly in the HBOC treated animals. In a related study Marchand et al. evaluated the effect of administering 0.5, 4, 10, or 30 ml/kg doses of DCLHb to unanesthetized swine bled 30 ml/kg (Marchand et al. 1996). DCLHb caused dose related increases in MAP and CO but at the lower doses the increase in CO was comparable to that seen in untreated control animals. Nevertheless, correction of base deficit and lactate concentrations was better in the HBOC treated animals.

To assess the effect of DCLHb resuscitation in animals with compromised coronary circulation, Habler and coworkers introduced a critical LAD stenosis prior to hemorrhage and resuscitation with HBOC or an oncotically matched HSA solution and assessed a number of cardiac specific parameters (Habler et al. 2000). DCLHb resuscitated animals exhibited higher survival (100 vs. 50 %), higher coronary perfusion pressures, and superior reversal of subendocardial ischemia and hypoxia. Furthermore, van Iterson and coworkers showed that the increased blood flow to the heart after DCLHb resuscitation resulted in increased epicardial oxygenation (Van Iterson et al. 2003). Preferential redistribution of blood flow to the heart has also been observed after DCLHb administration to hemorrhaged rats (Gulati and Sen 1998). On the other hand, a subsequent analysis of the Habler data suggested that left ventricular diastolic function may have been somewhat compromised even though myocardial oxygenation and animal survival were greatly improved (Pape et al. 2001). Also, in a combined traumatic brain injury (TBI) and hemorrhage model in swine, Malhotra et al. observed a 38 % incidence of mortality attributable to left ventricular failure in animals infused with a higher dose of DCLHb, compared to a lower dose of this HBOC or saline control animals (Malhotra et al. 2004).

A lengthy series of studies have been performed with HBOC-201 as a resuscitation solution, particularly in low volumes. In general this HBOC has resulted in improved survival and tissue oxygenation compared to resuscitation with standard crystalloid or colloid solutions, along with rapid increases in MAP, SVR and PAP (McNeil et al. 2001; Katz et al. 2002; Rice et al. 2006a). However, relative effects on CO have been mixed with some studies showing an increase in CO upon HBOC-201 administration, while others show a more diminished response. As discussed by Stern et al. these differences probably result from variations in traumatic insult, severity of hemorrhagic shock, fluid administration regimens, and

fluid types and volumes (Stern et al. 2009). These authors also emphasized that markers of tissue perfusion and oxygenation were often improved even in studies in which there was relative decrease in CO.

In hemorrhaged swine treated with PHP or MP4, CO was returned to baseline and MAP and SVR overshoot past baseline was less than that observed with other HBOCs (Noone et al. 1998; Drobin et al. 2004). However, there was an increase in PAP above baseline and compared to control solutions for both of these HBOCs.

### *33.3.4 Ischemia Reperfusion*

Given the fact that HBOCs will be used in compromised patients, regulators have strongly recommended that such formulations be evaluated in animal models that replicate stresses likely to be encountered in the clinic (Fratantoni 1991). One class of such models are those involving cardiac ischemia and reperfusion (I/R) injury. When Caswell et al. infused HBOC-201 into dogs 30 min prior to 90 min of occlusion of the left anterior descending (LAD) artery, followed by 270 min of reperfusion, there was a greater than 50 % reduction in infarct size compared to control animals infused with saline (Caswell et al. 2004). Myocardial blood flow was similar between the two groups during ischemia. Creatine kinase MB ($CK_{MB}$), a marker for cardiac tissue damage, was reduced in HBOC-201 treated animals in blood samples collected after 4 h of reperfusion, and neutrophil infiltration into the myocardium was also decreased. A substantial reduction in infarct size was also observed by George et al. when dogs received 1 g/kg HBOC-201 15 min after a constriction of the LAD artery sufficient to reduce blood flow by 80–95 % (George et al. 2006). Stress was enhanced by forced pacing of the heart at 110 % of the spontaneous heart rate. Ischemia was maintained for 195 min, followed by 180 min of reperfusion. Since HBOC infusion increased MAP and SVR, a reference group of dogs was titrated with phenylephrine (Phe) such that increases in blood pressure comparable to those observed upon HBOC infusion were replicated. No reduction in infarct size was observed in dogs infused with Phe, however, some reduction was observed in animals treated with $N^G$ –nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) production, suggesting that NO scavenging by the HBOC may contribute to the protective effect. Blood flow through the LAD artery was significantly improved after HBOC-201 infusion in contrast to the lack of increase observed after normal saline or Phe infusion. Regional myocardial function as assessed by sonomicrometric crystals placed in the region of ischemic tissue was restored to 92 % of baseline values in HBOC treated dogs after 15 min of reperfusion, compared to 11 and 49 % for animals infused with saline or Phe, respectively.

Positive results were also observed with the veterinary formulation of glutaraldehyde polymerized bovine hemoglobin (denoted as HBOC-200 by these authors) in cardiac I/R studies in rabbits and rats. When 0.4 g/kg of HBOC-200 was infused into rabbits 25 min prior to, or 10 min after the initiation of a 30 min

occlusion of a large marginal branch of the circumflex coronary artery, followed by 240 min of reperfusion, infarct area was reduced from 48 to 25 and 22 %, respectively (Rempf et al. 2009); however, areas of no reflow were comparable between treated and control animals. MAP increased and HR decreased in rabbits treated with HBOC-200. In rats, administration of 0.4 g/kg HBOC-200 prior to I/R resulted in a reduction of infarct area from 62 to 46 % after 25 min of occlusion of the left coronary artery followed by 120 min of reperfusion (Burmeister et al. 2005). No benefit was observed when HBOC was infused 10 min after the start of ischemia. The frequency of DNA single strand breaks was reduced in rats pretreated with HBOC relative to the positive controls, consistent with decreased tissue damage. Once again, MAP increased after HBOC administration, but in the pretreated animals the severity of cardiac arrhythmias was reduced during the ischemic period. The failure of HBOC treatment to alleviate infarct formation when administered after the initiation of ischemia in the rat study, as compared to the rabbit experiment, was noted by the authors, who are largely the same in both studies (Burmeister et al. 2005). One possibility suggested is that the duration of reperfusion varied between these experiments (Burmeister et al. 2005).

In a somewhat different protocol, Vandegriff and coworkers infused MP4 into rats subjected to a 30 min occlusion of the LAD, followed by 24 h of reperfusion (Vandegriff et al. 2008). The HBOC infusion was initiated 5 min prior to occlusion and continued throughout ischemia and reperfusion. Infarct size was comparable in vehicle treated control animals or rats infused with oxygenated MP4, in contrast to the results observed with HBOC-200 and HBOC-201. This may be attributable to the fact that MP4 has a much higher oxygen affinity ($P_{50}$ 5–6 mm Hg) (Vandegriff et al. 2003) compared to HBOC-200 ($P_{50}$ of 36 mm Hb) (Burmeister et al. 2005). Interestingly, treatment with MP4 preloaded with carbon monoxide did result in a statistically significant reduction of infarct size, an effect attributed by the authors as due to the protective effects of carbon monoxide.

Two studies have been published assessing the effect of HBOC infusion on myocardial ischemia during coronary angioplasty in swine. McKenzie et al. infused DCLHb solution (10 g/dL) at a rate of 40 mL/min through the lumen of a balloon angioplasty catheter placed in the proximal LAD coronary artery (McKenzie et al. 1994). Whereas balloon inflation in the absence of DCLHb infusion resulted in a decrease in MAP, $dP/dt_{max}$, and peak intraventricular pressure, and a significant S-T segment depression in the ECG, balloon inflation with DCLHb infusion resulted in an increase in MAP, $dP/dt_{max}$ and intraventricular pressure, and a diminished change in S-T segment depression. Thus, DCLHb infusion resulted in improved cardiac function during the ischemia caused by balloon inflation. Te Lintel Hekkert and coworkers evaluated the effect of infusing preoxygenated HBOC-201 at either 18 or 37 °C and flow rates ranging from 15 to 70 ml/min during 3 min occlusions of the proximal LAD (Te Lintel Hekkert et al. 2010). Optimal results were obtained using body temperature infusion at a rate of 50 ml/min which resulted in full preservation of left ventricular regional wall motion. HBOC treatment also inhibited the reactive hyperemia seen upon reinfusion and the purine washout which is indicative of a shift of heart muscle to

anaerobic metabolism. Perfusion with either nonoxygenated HBOC-201 or LR solution during occlusion had no salutary effect, even at the optimal flow rates. It was therefore concluded that the oxygen delivery by this HBOC supported normal myocardial aerobic metabolism and function during an LAD occlusion which would otherwise result in substantial deterioration.

## 33.4 Cardiac Lesions

During extensive preclinical testing of DCLHb, myocardial lesions were observed in certain species. This observation prompted a series of investigations to understand the pathogenesis and significance of this finding. This work has been reviewed elsewhere and will be summarized here (Burhop et al. 2004). The lesions are characterized as minimal to moderate, focal-to-multifocal myocardial degeneration and/or necrosis, often associated with an inflammatory infiltrate. The lesions were observed to varying degrees after single dose administration to cynomolgous, African green and rhesus monkeys, as well as pigs, with rhesus and pigs being the most sensitive species. The lesions were not observed after single infusions of DCLHb into dogs, rats or sheep, even at very high doses. Similar lesions were observed in rabbits but this species proved unsuitable as a testing vehicle because there was a significant background incidence in untreated and control animals. Time course studies in swine showed that degenerative myocardial changes appeared within hours of infusion, with morphological changes being most evident 24–48 h after treatment. Subsequently, necrotic tissue was removed and replaced in part by fibrous connective tissue and in part by enlargement of myocytes adjacent to affected areas. Some evidence of muscle fiber regeneration was also detected. Lesions therefore became much more difficult to detect at sacrifice intervals greater than a week after treatment. Dose response studies in both rhesus and swine demonstrated that only a small percentage of heart muscle was susceptible to the development of these lesions with no increase in lesion severity in the former above a DCLHb dose of 700 mg/kg. This was true even in repeat dose toxicity studies in which some animals received cumulative doses of 112,000 mg/kg DCLHb. Furthermore, many animals in these high dose studies did not exhibit histologic evidence of lesions, illustrating the high degree of capacity for cardiac tissue recovery. Morphometric analysis of rhesus hearts from animals treated and sacrificed to optimize development and detection of lesions showed an average of 1 % of the tissue was affected with a maximum involvement of 3 %. This extent of involvement would not be expected to affect cardiac function, a supposition that was supported by blinded ECG analysis of swine receiving 2 g/kg DCLHb or an oncotically matched HSA solution. No differences could be detected between control and treated animals, although after sacrifice the latter were demonstrated to have a 100 % incidence of lesions at a typical severity level. In addition, while transient elevations in lactate dehydrogenase (LDH) and creatine kinase (CK) enyzmes are consistently observed after DCLHb infusion to a variety

of species, the cardiac specific isoenzyme subsets of these enzymes were not relatively increased. This is consistent with the small amount of myocardium impacted by lesion development, but unfortunately eliminates these isoenzymes as surrogate markers for cardiac damage. Histologic analysis showed no evidence of endothelial pathology, nor was there any evidence of thrombus formation, in heart tissue exhibiting lesions.

In other experiments a number of aspects of lesion formation were investigated leading to the following observations:

- Chromatographic purification of DCLHb did not reduce lesion severity suggesting that contaminants are not responsible. This is also supported by the fact that lesion development is observed after infusion of human hemoglobin made in bacteria through recombinant technology, a source with a very different impurity profile than blood derived HBOCs.
- Lesions were observed in swine after the infusion of either human or swine stroma-free Hb, suggesting that formation is not an immunological response to the infusion of heterologous Hb, nor is it a result of the modification chemistry used to produce DCLHb.
- Lesions were not significantly reduced in incidence or severity by antihypertensives, anticoagulants, anti-inflammatory drugs, antioxidants, or the iron chelator deferoxamine.
- Similar lesions were observed whether DCLHb was administered as a volume load, a resuscitation solution, or in an isovolemic exchange transfusion protocol. Lesion development was also not affected by animal source, gender, hydration state, use of anesthesia, or catecholamine depletion.
- In contrast, lesion incidence and severity was reduced by polymerization of DCLHb with either glutaraldehyde or bifunctional polyethylene glycol reagents, suggesting that increasing molecular size was beneficial; however, the occurrence of lesions in rhesus could not be completely eliminated by the polymerization of DCLHb. Similar observations have been reported for other HBOCs. Only one instance of lesion formation has been reported after the preclinical testing of the polymerized bovine Hb HBOC-201 (Muir et al. 2011) and this was of low incidence and severity. Other histologic studies of this HBOC have reported no difference in heart findings between test and control animals (Rice et al. 2008), nor was any observed after standard toxicity testing (Rick Light, Greg Dubé, personal communication). In addition, no lesions were reported after infusion of 0.9 g/kg of MP4 into rhesus monkeys (Young et al. 2007). While MP4 is not polymerized, polyethylenglycol modification substantially increases the molecular size of this HBOC (Vandegriff et al. 2003).
- When a recombinant Hb tetramer with a 30-fold reduced rate of reaction with NO was evaluated, there was a marked reduction in overall lesion incidence and severity compared to tetramer with a rate of NO reaction comparable to human Hb. Furthermore, pigs infused with L-NAME, an inhibitor of NO synthase, developed cardiac lesions indistinguishable from those caused by DCLHb.

- The mitigating effects of polymerization and reducing the inherent rate of the NO reaction with oxyhemoglobin were additive. When a genetically produced Hb tetramer exhibiting a 30-fold reduction in NO scavenging was polymerized, the resulting product did not produce lesions in rhesus even at high doses.

The conclusion from this study is that the cardiac lesions observed after DCLHb infusion are a consequence of Hb extravasation into sensitive cardiac tissue and the subsequent reduction of local NO concentrations due to scavenging. While this is the same general mechanism by which HBOCs are believed to cause vasoconstriction, the phenomena differ in that cardiac blood flow is maintained or even increased after DCLHb infusion and lesion development does not appear to be a consequence of the increase in vascular resistance or increased blood pressure.

## 33.5 Effects of HBOCs on Human Cardiac Function

Several clinical trials have assessed the effect of HBOC infusion on cardiac function. HBOC-201 administration to elective surgery patients or patients prior to percutaneous coronary intervention (PCI) resulted in increases in MAP, SVR and pulmonary vascular resistance index (PVRI) and a concomitant decrease in CO (Kasper et al. 1998; Serruys et al. 2008). However, as observed in preclinical studies, overall oxygen consumption remained unchanged as a consequence of an increase in oxygen extraction ratio ($O_2ER$). Data collected from the more extensively instrumented PCI patients indicated that the left ventricular stroke work index (LVSWI) was not increased, and there was no evidence of a coronary artery vascular resistance increase (Serruys et al. 2008). Although limited to five patients, Meliga et al. evaluated the effect of HBOC-201 infusion directly into human coronary arteries immediately after the deployment of a stent (Meliga et al. 2008). Infusion was performed at a rate of 48 ml/min through the lumen of an angioplasty catheter during a 3 min arterial occlusion with the catheter balloon. During HBOC infusion, all measured hemodynamic variables were maintained at baseline levels, while there was a rapid decrease in ejection fraction, CO and minimal rate of left ventricular pressure change ($dP/dT_{min}$) and increase in end diastolic pressure when the balloon was inflated without HBOC perfusion. Premature termination of the occlusion before the 3 min time target was required for all five patients without perfusion and in none of the patients when infused with HBOC-201. No significant ST segment changes were observed during HBOC infusion, nor was there evidence of conduit artery coronary vasoconstriction. During anther clinical trial, infusion of HBOC-201 into a surgical patient with intra-operative myocardial ischemia increased MAP, decreased HR and rapidly normalized ST segment depression (Niquille et al. 2000). A subsequent postoperative episode of tachycardia and ST segment changes was also successfully treated by infusion of this HBOC. HBOC-201 was also used to treat an extremely anemic patient who was

severely injured but refused transfusion due to religious beliefs (Fitzgerald et al. 2011). HBOC infusion resulted in correction of ST depression, a decrease in troponin I levels and elimination of arrhythmias. These clinical studies indicate that cardiac blood flow and function is supported or even improved after the administration of HBOC-201, despite peripheral vasoconstriction.

Given the concerns about adverse cardiac effects of HBOCs, clinical trials with cardiac surgery patients are of particular interest. When HBOC-201 was administered to such patients in lieu of blood transfusion, there were modest changes in hemodynamic parameters and a decrease in CO that was again offset by an increase in $O_2ER$ (Levy et al. 2002). Arrhythmia was observed in 3/50 HBOC treated patients and 5/48 patients in the control group transfused with blood. When DCLHb was used instead of blood transfusion after cardiac surgery, small increases in MAP, SVR and PAP, and a clinically insignificant decrease in CO were observed relative to control patients. These changes did not negatively impact tissue perfusion as assessed by gastrointestinal function, base excess, blood pH and arterial lactate values (Lamy et al. 2000). Levels of $CK_{MB}$, lactate dehydrogenase-1 ($LDH_1$), and Troponin I were less than or comparable to those in blood treated patients. Two incidents of MI were reported in patients randomized to receive DCLHb, but it is unclear whether these events occurred before or after test article administration. In 28 anesthetized coronary artery bypass graft patients exchange transfused with increasing doses of Hb Raffimer, hypertension was more frequently observed than in control patients infused with HES, but oxygen delivery and oxygen extraction ratio did not differ significantly between the two groups of patients, nor did serum troponin I concentrations (Hill et al. 2002). Depending on the terms used to define serious myocardial adverse events, there were either five or seven such events in the Hb Raffimer treated patients, compared to two or seven in the 32 control patients. In a subsequent Phase III study, CABG patients exchange transfused with Hb Raffimer experienced nine MIs out of 148, versus five out of 151 control patients receiving pentastarch, a difference which was not statistically significant (Greenburg and Kim 2004). Elevations in $CK_{MB}$ and troponin I, and ECG changes diagnostic of myocardial ischemia, were very similar in both groups. As expected, hypertension was observed more frequently in HBOC infused patients, but this was readily managed. There was one death in the HBOC treated group and two in the control patients.

## 33.6 Discussion

In the four published studies in which HBOCs were infused into cardiac surgery patients, overall mortality was lower (8 vs. 12), while the incidence of MI was higher (12–19 vs. 7–12, depending on the criteria used for diagnosis) in treated versus control patients (Lamy et al. 2000; Hill et al. 2002; Greenburg and Kim 2004). While the former is encouraging, the latter is concerning, particularly in light of the fact that MI events are also imbalanced amongst other patient

subgroups (Greenburg and Pittman 2013; Silverman and Weiskopf 2009). On the other hand, side effects indicative of vasoactivity were generally mild and well managed in this patient population and enzymatic markers of myocardial damage were virtually identical between the test and control subjects. Furthermore, preclinical testing has shown that even when peripheral vasoconstriction is evident, blood flow to the heart is maintained or even increased and cardiac function is preserved, albeit sometimes with a decrease in CO.

The reason for the CO decrease, when it occurs, is debated. Increased afterload caused by the peripheral vasoconstriction is the most popular explanation (Irwin et al. 2008; Muir 3rd et al. 2000; Standl et al. 1997; Filho et al. 2005; Vaslef et al. 2001; Malhotra et al. 2004; Vane et al. 2002), but it has also been suggested that a CO decrease due to enhanced oxygen delivery to tissues may play a role (Muir 3rd et al. 2000; Standl et al. 1997; Stern et al. 2009; Kasper et al. 1998). The latter hypothesis is supported by a study in which dogs were partially exchange transfused with blood in which the Hb oxygen affinity was reduced, thereby facilitating oxygen release in tissues (Liard and Kunert 1993). The net result was an increase in SVR and a decrease in CO that is similar to that observed after HBOC infusion. Both of these mechanisms may be engaged after the infusion of HBOCs which scavenge NO and exhibit enhanced oxygen delivery. Also, as Stern et al. have noted, variable results have been obtained in different studies utilizing the same HBOC, which probably reflects variations in study design, severity of hemorrhagic shock insult (in resuscitation studies) and degree of volume depletion and repletion (Stern et al. 2009). In the latter regard, the dose response study of Marchand and coworkers is interesting in that low doses of DCLHb failed to restore CO after hemorrhage, while higher doses did so (Marchand et al. 1996). MAP was restored more rapidly and at lower doses. These results are explicable in light of the fact that vasoactivity is manifested at relatively low Hb doses, while volume repletion and expansion are more dependent on the amount of solution infused (Van Iterson et al. 2003). Rice et al. noted that greater volume repletion with HBOC-201 in a swine hemorrhage model resulted in better restoration of CO (Rice et al. 2006a). Insofar as HBOCs exhibit diminished vasoactivity and/or higher oncotic pressure, a more rapid restoration of CO would be expected.

It should also be noted that a decrease in CO is an issue only if tissue oxygenation is decreased and/or cardiac work is increased in compromised patients. In particular, Spahn and coworkers have expressed a specific concern about the use of HBOCs in patients with coronary artery disease due to concerns that their coronary arteries may be prone to exaggerated vasoactive responses (Spahn et al. 1994). As discussed above, numerous preclinical studies and several clinical studies in cardiac surgery patients have demonstrated that global oxygenation parameters are maintained or restored after HBOC infusion even when vasoactivity is manifested (Waschke et al. 1993; Standl et al. 1996; Standl et al. 1997; Dunlap et al. 1995; Marchand et al. 1996; McNeil et al. 2001; Stern et al. 2009; Kasper et al. 1998; Serruys et al. 2008; Lamy et al. 2000). In addition, the two clinical studies performed with PCI patients suggest that cardiac work is not increased after HBOC-201 infusion (Serruys et al. 2008; Meliga et al. 2008). Although a limited number

of patients were enrolled in these studies, it is encouraging that these results were obtained in patients who clearly have preexisting coronary artery disease. Even if increased cardiac work is required, this isn't necessarily problematic for those patients capable of such increases; however such demands may be an issue for those patients with highly compromised vasculature who are at the limits of their ability to compensate (Spahn et al. 1994). This may be especially troublesome if a vasoactive HBOC is combined with hypervolemia. Vane et al. noted in discussing their studies with hemorrhaged sheep that the hemodynamic effects of DCLHb may be exaggerated when the HBOC is administered after large volumes of lactated Ringer's solution, which may further limit the ability to increase CO (Vane et al. 2002).

The latter observation points to a limitation of many of the preclinical studies in that they were performed with healthy animals or animals subjected to a singular hemorrhagic or occlusive insult. The effects of chronic disease states, especially in combination, have not been well studied. In this regard the recent work by Yu and coworkers is interesting in showing that vasoactive responses to HBOC infusion are enhanced in rodents with endothelium altered by diabetes or a high fat diet (Yu et al. 2010). Biro has speculated that HBOCs may amplify adverse effects in endothelium damaged by hypertension, atherosclerosis and diabetes by increasing the nitroso-redox stress on these systems (Biro 2012). Another potential adverse combination may be the juxtaposition of damaged endothelium, HBOC and platelets. Platelet aggregation is known to be inhibited by NO, and, while HBOCs do not alter platelet function in vitro, platelet deposition on mechanically damaged endothelium was enhanced in an in vivo rabbit model (Radomski et al. 1990; Toussaint et al. 2003; Olsen et al. 1996). Pertinent to these considerations is an analysis of the adverse event profile of the use of HBOC-201 in a Phase III study in orthopedic surgery patients (Jahr et al. 2008). This analysis revealed there was a greater incidence of serious cardiac adverse events in treated patients who were elderly and also volume overloaded or anemic. These results suggest that the combination of vasoactive HBOCs, inherently fragile patients, and excessive or inadequate volume repletion should be more carefully evaluated in the formulation of clinical inclusion/exclusion criteria and patient treatment procedures. They also reinforce previous observations that historical criteria used to assess patient status may be inadequate when HBOCs are used as therapy (Driessen et al. 2001; Sampson et al. 2003; Rice et al. 2006b). For example, patients may have been appraised as well resuscitated when MAP, HR and central venous pressure were restored to the normal range, but in fact remained hypovolemic (Driessen et al. 2001; Rice et al. 2006b). Conversely, in other cases concern about lower CO and mixed venous oxygen saturation may have resulted in excess fluid administration (Sampson et al. 2003). Fluid overload may have also been exacerbated by the fact that HBOCs are even more potent volume expanders than originally anticipated (Fischer et al. 1999). Thus, it is currently unclear how much of the observed imbalance in cardiac serious adverse events in HBOC clinical trials represents a direct toxicity and how much is due to suboptimal treatment due to the unique characteristics of these products.

Another important question is whether the development of heart lesions such as those described after the administration of DCLHb to swine or primates could contribute to cardiac morbidity in humans. On the basis of currently available data, this possibility cannot be categorically excluded, however, it would seem unlikely for several reasons (Burhop et al. 2004). First, lesion formation is limited to only a small fraction of heart tissue and the maximum extent of involvement does not increase even when very large doses of HBOC are administered. Second, repair of the damaged area is efficient so that lesions are difficult to detect even histologically after a few weeks. Third, the lesions were subclinical in that they had no effect on cardiac function when present at maximum severity. Fourth, similar lesions are induced by clinically utilized catecholamines (Ballester et al. 1989; Kassim et al. 2008; Nixon et al. 2012). Fifth, polymerization or derivatization of Hbs significantly reduces the incidence and severity of lesion development as demonstrated by experiments with further modified DCLHb and the results of toxicity testing of HBOC-201 and MP4 (Muir et al. 2011; Rice et al. 2008; Young et al. 2007). Finally, results of ischemia/reperfusion experiments in swine have shown that the infusion of HBOCs supports cardiac function during ischemia and markedly reduces the extent of infarct development (Caswell et al. 2004; George et al. 2006; Rempf et al. 2009; Burmeister et al. 2005; McKenzie et al. 1994; Te Lintel Hekkert et al. 2010). Furthermore, HBOC infusion into a major conduit artery during balloon occlusion, supports normal coronary function (Kasper et al. 1998; Serruys et al. 2008; Meliga et al. 2008). Thus, unless humans are much more sensitive to lesion development than the most sensitive other species identified to date, it is unlikely that this mechanism is of clinical significance.

In summary, no specific cardiac toxicity mechanism has been identified in humans and the reasons for the MI imbalances observed in some human clinical trials remains unclear. It is likely to be multifactorial. While generalized "vasoactivity" has been blamed, this is inconsistent with preclinical data which show that blood flow and cardiac functionality are well maintained after HBOC administration, and clinical results that show that direct infusion of HBOC into major human coronary arteries does not result in their vasoconstriction. If there is a direct cardiac toxicity of clinical significance, its cause is likely to be more subtle than a generalized vasoactive response, and to discern this we must tease out a number of confounding factors. It is possible that increased cardiac demand caused by peripheral vasoactivity may be a factor in a highly compromised subset of patients, but the clinical and preclinical data suggest that it is at least as likely that some of these patients were volume overloaded, volume depleted, or anemic due to suboptimal treatment. This emphasizes the need for careful future assessment of the effect of HBOC administration on blood flow and tissue oxygenation in human cardiac vasculature. Ideally, the use of noninvasive technologies would permit such assessments on a wide range of patient types so that cardiac effects of HBOCs could be separated from those resulting from other factors and so that the interaction of HBOCs with a variety of preexisting conditions, such as compromised endothelium, could be assessed. Given the potential of HBOCs to address a range of unmet medical needs, this would be a worthy undertaking.

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# Chapter 34
# Effects of Hemoglobin-Based Oxygen Carriers on Blood Coagulation

**Jonathan S. Jahr, Molly Chung, Afsaneh Anvarhosseini and Hae Won Kim**

## 34.1 Introduction

There have been at least two major concerns with the use of HBOCs as resuscitation fluids for traumatic hemorrhage: potential coagulopathy and interference with conventional clinical laboratory coagulation measurements. These concerns regarding coagulopathy with HBOCs have long been articulated, as has interference regarding HBOCs and coagulation laboratory equipment not designed for plasma hemoglobins (Jahr et al. 2002).

Possible mechanisms are postulated that include the large molecular size of some HBOCs, as seen with hetastarches and gelatins, nitric oxide scavenging and interference with platelets, and formation of methemoglobin by oxidation of the HBOC and consequent coagulation aberrations (Moallempour et al. 2009). Additional etiologies include decreased plasma calcium concentrations, secondary to volume expansion with non calcium containing solutions.

Hemorrhagic shock and trauma are the leading cause of death in battlefields. Most deaths occur during the first hour from injury, as a result mortality due to trauma and severe hemorrhage may be greatly reduced by early fluid resuscitation. Traumatic hemorrhage associated coagulopathy is partly due to the consumption of both coagulation factors and platelets and partly due to dilutional effect of resuscitative fluids. Hypothermia and acidosis resulting from injury also interfere with clot formation. When a vessel is injured, sequential activation of the coagulation cascade and platelet aggregation results in the formation of hemostatic plug

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at the site of injury to prevent further bleeding, but when bleeding is severe, consumption of coagulation factors followed by infusion of large volume resuscitative fluids devoid of clotting factors and platelets may result in complications such as disseminated intravascular coagulation (DIC), and multi organ failure (Arnaud et al. 2007). Studies suggest that in hemodiluted conditions as such, addition of low doses of recombinant factor VII (rFVIIa) combined with other procoagulants might be of benefit due to its involvement in activation of both intrinsic and extrinsic coagulation mechanisms. Recombinant factor VIIa has been used off-label in combat casualties with severe hemorrhage in an attempt to decrease mortality and the need for blood transfusions (Darlington et al. 2011).

Dilutional coagulopathy with common resuscitative fluids is strongly correlated with the degree of hemodilution regardless of the fluid type used. Studies suggest that the greatest dilutional coagulopathy occurs at hemoglobin less than 6 G/dL, platelets less than 100,000 and fibrinogen concentration at less than 200 mg/dl, threshold indeces that maybe of great value in determining when to transfuse blood or blood products in critically ill patients (Darlington et al. 2011).

Issues specific to blood transfusions, such as transfusion transmitted infections, transfusion reactions, compatibility issues and world wide shortage of donor blood, has led to the development of blood substitutes. Artificial blood substitutes have been the focus of many studies for many decades, in the hope of developing clinically useful products that are universal oxygen carriers with minimum toxicity to manage patients with difficult to control bleeding, especially those situated in difficult to reach areas such as combat zones, mines, etc., where sophisticated medical care, including transfusion of blood or blood products, is not available (Kim and Greenburg 2006; Feola et al. 1988). A number of products have been studied and many of them had to discontinue production due to safety issues including vasoconstriction thought to be related to NO scavenging, GI symptoms, cardiac problems, nephrotoxicity, and death (Johnson and Swiatkowski 2007). Two major issues of the earlier generations of unmodified cell free hemoglobin in HBOCs were their high affinity for oxygen, due to loss of 2,3-diphosphoglycerate (DPG) during purification, and a short intravascular retention time (Kim and Greenburg 2006). To correct these issues led to the production of polymerized (cross-linked) and conjugated hemoglobin/heme based oxygen carriers (Feola et al. 1988). Human gluteraldehyde polymerized hemoglobin (PolyHeme®, Northfield Laboratories, Chicago, IL), HBOC-201 (Hemopure® Biopure Corp/OPK Biotech., Cambridge, MA), and MP4 (Hemospan®, Sangart Corp, San Diego, CA) have been studied in advanced clinical trials to prove safety and clinical benefits to be used as a universal blood substitute in critical care patients. Encapsulated Hbs and perfluorocarbon based oxygen carriers are also in development. HBOCs with built in antioxidants have been developed to reduce oxygen radical mediated damage. Also, with recent advances in recombinant DNA technologies, recombinant HBOCs have been developed and may be modified many ways to achieve desired results (Kim and Greenburg 2006).

A distinct advantage of HBOCs over standard fluids lies in the capability of supplying oxygen to tissues and the lower volume needed to maintain hemostasis

compared to crystalloid solutions in trauma patients with severe hemorrhage with delay in access to hospital care. This may allow ample time for a safe evacuation and transportation of wounded to where definite care is available (Arnaud et al. 2007). Other advantages of red cell substitutes include long shelf life without refrigeration with no type and cross-match requirements. This may result in a better clinical outcome in patients who require immediate transfusion but are situated where blood is not available (e.g., battle fields). If approved, the lower cost, ease of use and universal compatibility of HBOCs will not only change transfusion medicine in USA, but also will greatly affect transfusion medicine all over the world, since 80 % of the world population lives in areas where safe blood or blood products are not available (Kim and Greenburg 2006).

A number of compounds have been studied for coagulation issues, including a number of products no longer available: diaspirin crosslinked hemoglobin, (DCL-Hb/HemAssist®, Baxter), hemoglobin raffimer (Hemolink®, Hemosol), PolyHeme®, (human gluteraldehyde polymerized hemoglobin, Northfield Laboratories). Two products are commercially available from OPK Biotech (formerly Biopure), Cambridge, MA: HBOC-200 (Oxyglobin®, hemoglobin glutamer-200 (bovine), and HBOC-201 (Hemopure®, hemoglobin gluatamer-250 (bovine). A newer product, a zero-linked polymerized hemoglobin, (Oxyvita®, OXYVITA, Inc.), which is currently in preclinical testing, has also been assessed for its potential to cause coagulation abnormalities (Arnaud et al. 2007; Jahr et al. 2012).

This chapter be divided into the following sections: introduction, review of preclinical and clinical coagulation studies with stroma-free hemoglobin (SFH) and other hemoglobin-based oxygen carrier (HBOC) products, coagulation pathways, coagulation testing and commonly used instruments, HBOC effects on platelet function, HBOC and coagulation assessment, potential mechanisms, establishment of need for oxygen delivering resuscitation fluids, potential approaches to development of improved resuscitations fluides, and discussion and summary.

## 34.2 Review of Preclinical and Clinical Coagulation Studies with Stroma-Free Hemoglobin and Other Hemoglobin-Based Oxygen Carrier (HBOC) Products

Recent generation HBOCs are often evaluated for coagulopathy by TEG as it is a measure of whole blood coagulation. Older studies with stroma-free hemoglobin, and 1st and 2nd generation HBOCs often utilized traditional coagulation tests such as protime (PT), which measures the extrinsic pathway, and activated prothrombin time (aPTT), which measures the intrinsic pathway. Results of older studies are difficult to compare with those with more current HBOC products because they demonstrated varying results, in part due to a large variability in experimental design and less well characterized HBOC preparations used.

Stroma-free hemoglobin (SFH) had a mild prolongation of aPTT (Savitsky et al. 1978). Transfusions of 500 ml of 5–6 % SFH solutions led to a slight decreasing of fibrinogen concentration and activity of clotting factors V, VII, VIII, IX, and X and a prolongation of thrombin time (Uszynski et al. 1976). The prolongation of thrombin time may reflect disturbed polymerization of fibrin. Stroma-free hemoglobin was also studied in vitro and in vivo in rabbits (Browdie and Smith 1975) showing no difference in prologation of PT and aPTT compared to saline. In another study, neither SFH nor polyhemoglobin caused significant changes in PT, aPTT, factor X, fibrinogen, antithrombin III, and antiplasmin levels in rats (Ning and Chang 1990).

Hemoglobin vesicles (Abe et al. 2006), hemoglobin encapsulated in a liposome, were shown to prolong PT and aPTT above the normal range at mixing ratios of 60 %. Complement titers were also examined, and the pegylated hemoglobin vesicle showed less consumption of complement compared to the unpegylated vesicle. It is thought that negatively charged liposomes activate complement and triggers the intrinsic coagulation pathway, but pegylation seemed to decrease complement activation. Polymerized bovine hemoglobin (HBOC-200) had no effect on PT or PTT in ponies (Belgrave et al. 2002). In patients undergoing liver resection, hemodilution with HBOC-201 and 6 % hetastarch did prolong APTT from baseline, but there was no difference between HBOC-201 and 6 % hetastarch (Standl et al. 1998). Pyridoxalated PEG hemoglobin in vitro showed a tendency to prolong thrombin time similar to the saline control (Iwasaki et al. 1986). Albumin-heme in vitro showed no effect on PT and PTT (Huang et al. 2003).

## 34.3 Coagulation Pathways

The process of preventing blood loss from a vessel or organ of the body is referred to as hemostasis (Lefkowitz 2008). Major constituents of hemostatic system are endothelium, platelets and coagulation factors, which interact and work with each other with the ultimate goal of clot formation and preventing blood loss at the site of an injury, while at the same time controlling clot extension beyond the injury site by antithrombotic mechanisms and finally by clot removal once healing and repair is complete (see Fig. 34.1 for Coagulation Overview (Lefkowitz 2008)).

Vasoconstriction of blood vessels occurs as a first response to blood loss and primary hemostasis takes place by platelet plug formation (Lefkowitz 2008). Von Willebrand Factor secreted from endothelial cells acts as an intercellular glue, binding platelets to one another and to damaged endothelium and forms a plug that temporarily seals the break in the vessel wall (see Table 34.1 for Coagulation Factors (Lefkowitz 2008)).

Secondary hemostasis takes place simultaneously by the formation of a fibrin clot (Lefkowitz 2008). Proteins in the blood plasma, called coagulation factors

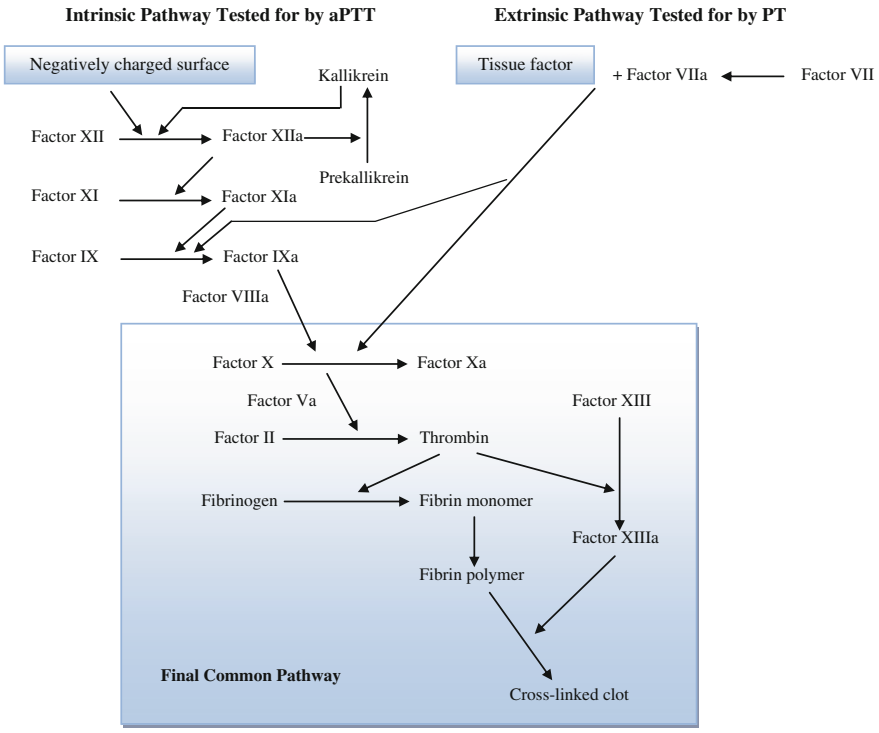


**Fig. 34.1** Hemostasis physiology. Modified from Shore-Lesserson L, Committee on Blood Management Monograph on Platelets, American Society of Anesthesiologists, 2012

(Table 34.1), respond in a complex cascade to form fibrin clot, which strengthen the platelet plug. The coagulation cascade has two pathways, the contact activation pathway: Intrinsic Pathway, and the tissue factor pathway: Extrinsic Pathway (see Fig. 34.1). The primary pathway for initiation of coagulation is the extrinsic pathway. Extrinsic pathway mainly generates small amounts of thrombin, the main constituent of the coagulation cascade and thrombin in turn by its feedback activation role, through an intrinsic mechanism generates more thrombin, which in turn forms the definitive clot at the sit of injury.

The intrinsic pathway is initiated by activation of the "contact factors" and can be measured by the activated partial thromboplastin time (aPTT) test (Lefkowitz 2008). The extrinsic pathway is initiated by tissue factor and can be measured by prothrombin [PT] test. The classic coagulation path way is based on in vitro testing and might not be a correct representation of blood clotting in vivo. Various factors are needed for the proper functioning of the cascade such as calcium and phospholipid. Vitamin K is also essential for synthesis of factors II, VII, IX, and X (Lefkowitz 2008).

**Table 34.1** Clotting factors: name, description, and function

| Name | Description | Function |
|---|---|---|
| Fibrinogen (Factor I) | Molecular Weight (MW) = 340,000 daltons (Da); glycoprotein | Adhesive protein that forms the fibrin clot |
| Prothrombin (Factor II) | MW = 72,000 Da; vitamin K-dependent serine protease | Activated form is main enzyme of coagulation |
| Tissue factor (Factor III) | MW = 37,000 Da; also known as thromboplastin | Lipoprotein initiator of extrinsic pathway |
| Calcium ions (Factor IV) | Necessity of $Ca^{++}$ ions for coagulation reactions described in 19th century | Metal cation necessary for coagulation reactions |
| Factor V (Labile factor) | MW = 330,000 Da | Cofactor for activation of Prothrombin to thrombin |
| Factor VII (Proconvertin) | MW = 50,000 Da; vitamin K-dependent serine protease | With tissue factor, initiates extrinsic pathway |
| Factor VIII (Antihemophilic factor) | MW = 330,000 Da | Cofactor for intrinsic activation of factor X |
| Factor IX (Christmas factor) | MW = 55,000 Da; vitamin K-dependent serine protease | Activated form is enzyme for intrinsic activation of factor X |
| Factor X (Stuart-Prower factor) | MW = 58,900 Da; vitamin K-dependent serine protease | Activated form is enzyme for final common pathway activation of prothrombin |
| Factor XI (Plasma thromboplastin antecedent) | MW = 160,000 Da; serine protease | Activated form is intrinsic activator of factor IX |
| Factor XII (Hageman factor) | MW = 80,000 Da; serine protease | Factor that normally starts aPTT-based intrinsic pathway |
| Factor XIII (Fibrin stabilizing factor) | MW = 320,000 Da | Transamidase that cross-links fibrin clot |
| High-molecular-weight kininogen (Fitzgerald, Flaujeac, or William factor) | MW = 110,000 Da; circulates in a complex with factor XI | Cofactor |
| Prekallikrein (Fletcher factor) | MW = 85,000 Da; serine protease | Activated form that participates at beginning of aPTT-based intrinsic pathway |

Modified from Lefkowitz 2008

## 34.4 Coagulation Testing and Commonly Used Instruments

Two major methods are utilized for detection of coagulation testing: optical or mechanical clot detection. Theoretically, optical may be interfered with if there is a hemolyzed blood specimen or in the case of HBOCs, plasma hemoglobin. Coagulation testing can be delineated by the characteristics of the clotting cascade being assessed (see Table 34.2). Table 34.2 also describes instrument specific methods for detection.

Mechanical instruments may be less susceptible to interference from HBOCs but each instrument/methods must be validated so that accurate clinical interpretation can be made. For example, only the fibrometer was shown to be reliable with HBOC-201 when its concentrations in the sample is greater than 4.8 g/dL (Jahr et al. 2002).

Among the instruments described in Table 34.1, three instruments utilize mechanical clot detection and five instruments use optical clot detection methods for determining prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen (FBG). Antithrombin (AT) values may also be calculated using chromogenic principles on devices that are capable of performing this assay method.

Devices based on thromboelastography or thromboelastometry such as TEG (Haemoscope, Niles, IL (TEG 2005)) and ROTEM (Tem Innovations GmbH, Munich), can monitor the entire process of blood coagulation process using a whole blood sample rather than a plasma sample. Therefore, it can assess qualitative state of hemostasis process that depends on quantity as well as functional status of platelets, plasma clotting factors and fibrinolytic system.

Platelet function can be assessed using thromboelstographic devices. For example, TEG can be used to assess platelet function by utilizing different anticoagulants and differential PLT activators. For example, a standard citrated blood sample with kaolin elicit no effects on PLT function and heparinized blood sample represent blood clot soley based on fibrin as heparin inhibits thrombin and PLT participation. Heparinized blood with ADP (for ADP inhibitor users) or arachidonic acid (for aspirin users) activates PLTs not affected by the drugs (Squires 2002). TEG variables include R (reaction time; time to clot initiation), K and $\alpha$ (rate of clot development), MA (maximum amplitude), and G (maximum clot strength). The measurement R is defined as the latency period between placement of blood in the TEG analyzer and start of clot formation. MA defines the strength of the fibrin/platelet/factor XIII cross-linked aggregate. G is a transformation of MA into units of kdyn/cm2. While both MA and G are reported, G enables results from individual experiments to be more directly compared. The normal range of TEG using sodium citrate and celite/kaolin based on a study of 98 volunteers is reported as: R (2–8 min), K (1–3 min), $\langle(55^{o} - 78^{o})\rangle$, MA (51–69 mm), and G (4.6–10.9 kdyn/cm2) (TEG 2005).

**Table 34.2** Coagulation testing

| Instrument | Mechanism | Clinical Utility |
|---|---|---|
| Thromboelastograph® | Viscoelastic/Thrombin<br>PlateletMapping: ADP, AA | Thrombocytopenia, and anti-GPIIb/IIIa therapy<br>PlateletMapping for aspirin and $P2Y_{12}$ inhibitors |
| PFA-100® | Closure of a membrane aperture/collagen + ADP, or collagen + epinephrine | vWD, congenital platelet disorder, aspirin therapy |
| Diagnostica Stago (Parsippany, NJ) | Electromechanical clot detection system; chromogenic capabilities for AT measurements. | PT, aPTT, FBG, and AT |
| BBL Fibrometer (Becton–Dickinson, Baltimore, MD) | Fibrometer, mechanical clot detection | PT and aPTT |
| Cardiovascular Diagnostics (Raleigh, NC) | Single-use disposable test cards using the Thrombolytic Assessment System; optical system | PT and aPTT |
| Dade Behring (Miami, FL) | Optical clot detection; chromogenic capabilities for AT testing | PT, aPTT, FBG, and AT |
| Hemoliance (Raritan, NJ) | Optical clot detection; chromogenic capabilities for AT testing. | PT, aPTT, FBG, and AT |
| Organon Teknika Corporation (Durham, NC) | Optical detection system; may correct for lipemia and hemolysis | PT, aPTT, FBG, and AT |
| Sigma Diagnostics (St. Louis, MO) | Optical clot detection method (Sigma-O) or mechanical clot detection method (Sigma-M) | PT, aPTT, FBG, and AT |

Mechanical instruments may be less susceptible to interference from HBOC-201; however, only the fibrometer should be reliably used for PT and aPTT testing with HBOC-201 concentrations greater than 4.8 g/dL (Jahr et al. 2002).

Platelet function may also be measured using the PFA-100 system (Dade Behring, Marburg, Germany). This is a rapid screening tool that is more sensitive in detecting major platelet function defects than the standard bleeding time measurement method (Favaloro 2002). The PFA-100 models high shear stress in vivo conditions by using a capillary apparatus to measure platelet function. Closure time (CT) is defined as the time when blood ceases to flow through the apparatus, implying the formation of a platelet thrombus. Two types of platelet agonists are used to coat the collagen cartridges, epinephrine (cEPI) or adenosine diphosphate (cADP) (Favaloro 2002; Mammen 1998). Nonclosure is defined as CT greater than 300 s. The normal reference ranges were 71 to 118 s for cADP and 94 to 193 s for cEPI (Favaloro 2002). The cEPI cartridge is more sensitive compared to the cADP cartridge, and is especially sensitive to drug-related platelet dysfunction (Newark and Inc 2005; Harrison et al. 2002).

## 34.5 HBOC Effects on Platelet Function

Platelet function is an important determinant of coagulation. The effect of various HBOCs on platelet function has been studied ex vivo, in animal models, and in humans. In hetastarch solutions, coagulopathy has been shown to correlate with the content of high molecular weight polymers present (Strauss et al. 2002). Studies have suggested that the mechanism involves hetastarch binding to coagulation factors and the surfaces of red blood cells and platelets leading to accelerated clearance of coagulation factors and decreased platelet activation (Huraux et al. 2001; Deusch et al. 2003; Weeks et al. 2008).

As HBOCs are high molecular weight molecules, studies have been done to assess their effect on coagulation. An ex vivo model compared the effects of two HBOCs of different molecular weights, Zero-linked Hb polymer (33 megadaltons) and HBOC-200 (200 kilodaltons), with 6 % hetastarch (670 kilodaltons) on coagulation by thromboelastography (TEG) (Jahr et al. 2008a). TEG analysis of clotting parameters R, K, MA, and G were studied in whole blood diluted by 6 % hetastarch, HBOC-200, or Zero-linked Hb polymer at low (1:11), medium (1:5), high (1:2), and very high (1:1) dilutions. There were no significant differences between Zero-linked Hb polymer and HBOC-200 in any aspect of coagulation at any dilution, despite their difference in molecular weight. This suggests that greater coagulopathy is not inherent with extensive polymerization in HBOC products. Compared to 6 % hetastarch at low and medium dilutions, both Zero-linked Hb polymer and HBOC-200 showed no difference in effect on clot strength (MA, G). However, at high and very high dilutions, Zero-linked Hb polymer and HBOC-200 products exhibited a decrease in clot tensile strength by 33 and 49 %, respectively, compared with 6 % hetastarch. While the mechanism for this difference has not been elucidated, use of Zero-linked Hb polymer at the small volumes recommended by the manufacturer (2–3 ml/kg), which corresponds to a

dilution less than the 1:11 "low dilution", should not increase risk of clinical bleeding.

In an animal model of severe uncontrolled hemorrhage, markers for coagulopathy including platelet function analyzer closure time (PFA-CT) and TEG were compared among groups resuscitated with HBOC-201 or Hextend® (HEX) and a nonresuscitated group (Arnaud and Handrigan 2006). PFA-CT for HBOC-201 and HEX were higher compared to the nonresuscitated group. The peak PFA-CT occurred at 4 h for the HEX group compared to 24 h for the HBOC-201 group. By 48 h, PFA-CT was back to baseline in all groups after blood transfusion. The time to clot formation (TEG-R) was increased in HBOC-201 animals at 24 h compared to HEX animals. Clot strength (TEG-MA) decreased in the HEX group, and to a lesser extent in the HBOC-201 group when compared to the nonresuscitated group. While the nonresuscitated group experienced a post-hemorrhage coagulation pattern that allowed rapid control of bleeding, as evidenced by TEG and PFA-CT parameters as well as prothrombin time, this group also suffered a higher level of mortality. In this study, resuscitation with HBOC-201 did produce a mild dilutional coagulopathy, similar to resuscitation with HEX, when compared to no resuscitation.

An in vivo human study compared platelet function before and after transfusion with either HBOC-201 or packed red blood cell (PRBC) in an adult population undergoing elective orthopedic surgery (Jahr et al. 2010). The PFA-100 system was used to measure closure time. Closure time was measured before transfusion and at several time points after transfusion. In the PRBC group, closure time did not significantly change at any time point. In the HBOC-201 group, closure time was significantly prolonged after transfusion, reaching levels above the upper limit of normal but below the nonclosure time. At Day one after transfusion, closure time for the HBOC-201 group returned to within the normal range. This study echoes the ex vivo and animal studies in illustrating that HBOCs produce mild platelet dysfunction. The clinical significance of these findings, however, is unclear as severe bleeding from thrombocytopenia is rare without concomitant coagulopathy or other defects in the vascular system.

Another mechanism proposed for platelet dysfunction is oxidation of the cell-free hemoglobin in HBOCs to methemoglobin. These oxidative products modify redox-sensitive sites involved in platelet aggregation and activation. In an ex vivo study (Moallempour et al. 2009), previously opened packages of HBOC-200 reached 65 % methemoglobin concentration compared to a 1 % methemoglobin concentration in freshly opened bags. Measuring TEG parameters showed statistically significant impairment in clot propagation and strength in the high methemoglobin samples. This study suggests that HBOC may affect coagulation beyond dilutional effects if methemoglobin is allowed to accumulate.

## 34.6 HBOC and Coagulation Assessment

Apart from platelet function, coagulopathy is also mediated by changes in the coagulation cascade, commonly measured by PT, aPTT, and AT. The increased hemoglobin concentration in HBOC-201 may interfere with the performance of coagulation analyzers commonly used for PT, aPTT, and AT assays. An in vitro study compared HBOC-201-prepared plasma samples at different concentrations with saline-prepared samples on various coagulation analyzers (Jahr et al. 2002). Interference by HBOC-201 was defined as a difference of >10 % from control. The data showed that at low concentrations of HBOC-201(2.6 G/dL), all instruments (optical and mechanical) reached an accurate and satisfactory result. However, at higher concentrations (3.8 G/dL), optical instruments gave inaccurate results. In these higher concentrations of HBOC-201, the only reliable method was the fibrometer. This demonstrates the importance of choosing the appropriate analyzer when using HBOCs to evaluate for coagulopathy. This will be covered in more detail in the chapter by Smani, et al.

In animal studies, the effect of resuscitative fluids HBOC-201 and a high molecular weight hydroxyethyl starch (Hextend®, Biotime Inc., Berkely, CA) on coagulation was compared to a nonresuscitated group after severe controlled bleeding and a delay to hospital care of 4–24 h. The study showed that the delay of 4–24 h resulted in a lower survival rate for the nonresuscitated group while survival was similar between the HEX and HBOC-201 groups (Arnaud et al. 2007). Coagulopathy was evident in both resuscitated groups due to the dilutional effect of these fluids given in the pre-hospital phase, compared to hemoconcentration in the nonresuscitated group. Transfusions with blood products were given in the hospital phase of the study and showed correction of coagulopathy. Due to the decreased need for blood transfusion in the HBOC-201 group, lab parameters indicating coagulopathy was noted to be higher in this group compared to the other two.

The effect of HBOC-201 (Hemopure®) on different coagulation analyzers has been mostly attributed to interference due to the color change in plasma rather than by the molecule itself. A study designed to further investigate the effect of HBOC-201 on coagulation compared blood samples diluted with either HBOC-201 or lactate ringers (LR) (James 2004). Thromboelastograph (TEG) parameters were analyzed. The advantage of using TEG over common lab methods of measuring coagulation, such as (PT) and (aPTT) is that TEG is a measure of whole blood coagulation not just plasma. At concentrations of 2 g/dl both HBOC-201 and (LR) solutions showed significant decrease in reaction time as well as coagulation times and an increased alpha angle. Therefore, it seems that HBOC-201 and (LR) have similar dilutional effects on coagulation at the comparable clinically relevant concentrations.

## 34.7 Potential Mechanisms

Many studies have simulated hemodilution during clinical resuscitation of hemorrhagic shock with increasing doses of HBOCs compared to crystalloid or colloid (e.g., hetastarch) fluids. These results suggest a dilutional coagulopathy that seems to be shared by all the above products. For example, when Zero-link Hb polymer, an ultra-high molecular weight HBOC, HBOC-200, 6 % hetastarch, and 0.9 % normal saline were used to dilute whole blood (Jahr et al. 2008a), progressive dilution showed a decrease in TEG parameters for clot strength (MA) and a prolongation of clot kinetics (α). Though the results did not reach statistical significance, crystalloid, colloid, and Zero-link Hb polymer tended to shorten R (clot initiation) at lower dilutions followed by gradual lengthening with higher dilutions. This initial hypercoagulable effect is thought to be mediated by a greater sensitivity of anticoagulants such as antithrombin III to the effects of mild dilution compared with coagulation factors, thrombin, and other factors in the coagulation cascade. This effect has been further described in studies comparing various crystalloids and colloids at increasing dilutions. Up to a 40 % dilution for colloids and 50 % for crystalloids, there was an increase in coagulability (increase in speed of clot formation and clot strength). However, after 70 % dilution, there was evidence of hypocoagulation (Ekseth et al. 2002).

In another study, in vitro dilution with normal saline, polyhemoglobin solution, 5 % bovine albumin at 25 and 50 % dilution did not change TEG parameters r and k significantly (Kim et al. 1992). Stroma-free hemoglobin did show a moderate procoagulant trend at 25 and 50 % dilution with shortened r and k times. A proposed mechanism for this is that stroma-free hemoglobin may be less stable in solution leading to auto-oxidation of hemoglobin to methemoglobin, thereby releasing superoxide radicals that may initiate procoagulant processes such as cell damage and platelet activation. The clot strength parameter MA did decrease in all groups with increasing dilution, suggesting again a mechanism of dilution. Since clot strength does seem to be correlated with hematocrit and platelet counts (Kim et al. 1992), (Table 34.3) a dilutional explanation for this variable is a consistent interpretation.

When low and medium weight hydroxyethyl starches were similarly compared, there was no difference in platelet count or fibrinogen concentration (Jamnicki et al. 2000). However, there was a decrease in factor VIII and von Willebrand

**Table 34.3** Potential mechanisms for coagulopathy

| Potential mechanisms for coagulopathy with hemoglobin based oxygen carriers |
|---|
| 1. Dilutional coagulopathy/hypocalcemia |
| 2. Oxidation to methemoglobin inhibiting platelet aggregation |
| 3. Large molecular weight molecules complexing with von Willebrand factor and speeding its elimination |
| 4. Nitric oxide scavenging |

factor (vWF) activity that was more pronounced in the medium weight fluid (HES200) compared to the low weight fluid (HES70). There was also greater increase in aPTT and a more pronounced decrease in MA and α by HES200 compared to HES70. Since these two TEG parameters are influenced by platelet count and function, and there was no difference in platelet count between the HES solutions, the difference may be due to the more pronounced decrease in vWF and its role in platelet linking. The most likely mechanism for the decrease in vWF is enhanced elimination. It is proposed that larger HES molecules may form complexes with the vWF to be cleared from the circulation. Similarly, the greater increase in aPTT may be a reflection of the greater decrease in vWF as well as in factor VIII by the larger molecular weight fluid. As HBOCs are also large molecular weight compounds, there is theoretical concern that similar mechanisms may be present with HBOCs.

Other studies show similar though slightly varying results. HBOC-201, approved in South Africa for treatment of adult surgical patients, was tested in vitro against lactated Ringer's solution and showed no significant difference in TEG parameters at clinically relevant concentrations (James 2004). Whole blood was diluted with HBOC-201 and LR, and at concentration of 2 g/dL, both fluids showed statistically significant shortened R and K times with an increased α angle compared to undiluted control samples. These results suggest a possible procoagulant effect at a HBOC-201 concentration of 2 g/dL, which corresponds to about 20 % hemodilution. In contrast to the above studies, MA did not vary significantly from undiluted control in any of the tested samples. However, the only statistically significant difference between HBOC-201 and LR in this study was a slight decrease in MA by HBOC-201 compared to LR which marginally increased MA. MA reflects the properties of platelets, fibrinogen and factor XIII in contributing to clot strength, so this may reflect a minor effect on platelet function by HBOC-201.

Similarly, in a swine model of hemorrhagic shock (Arnaud et al. 2005), animals were resuscitated with HBOC-201, hydroxyethyl starch or not resuscitated. Parameters for clot kinetics, TEG-R, K, and α were likewise increased while clot strength (MA) was decreased. This study also showed an increase in PFA-CT in the HEX and HBOC group. PFA-CT is increased by low platelet count, qualitative platelet defects, and qualitative/quantitative vWF deficiencies. Notably, PFA-CT is unaffected by coagulation factor deficiencies and hypofibrinogenemia. As the control group did not differ from the HEX and HBOC group in total number of platelets, this result suggests hemodilution of vWF plays a role in the coagulopathic effects of hemodilution with HBOC-201.

Another mechanism posited for abnormal coagulation with HBOC is nitric oxide (NO) scavenging by free hemoglobin resulting in increased hemostasis. NO is synthesized by the vascular endothelium and acts to relax vascular smooth muscle as well as inhibit platelet activation. Hb raffimer or O-raffinose cross-linked hemoglobin (Hemosol, Inc.) decreased bleeding time in anemic and thrombocytopenic rabbits compared to albumin (control) and phenylephrine infusion (to assess vasoconstriction effect comparable to Hb raffimer) (Lee et al. 2000). The in vivo studies in this paper suggest that Hb raffimer did enhance aggregation of

stimulated platelets by abolishing the inhibitory effect of NO. Furthermore, the in vivo study showed a decreased time to carotid artery occlusion by a platelet-rich thrombus in rabbits given Hb raffimer.

However, in an in vitro study with human whole blood, Hb raffimer showed no apparent effect on platelet activation and function. Addition of Hb raffimer to blood samples up to 50 % volume did not cause platelet activation as measured by various markers of platelet activation (CD42b, CD41, PAC-1, CD62, CD63, annexin V and microparticle formation) and PFA-CT (Leytin et al. 2003).

Based on available data (Jahr et al. 2008b; Williams et al. 2002), it appears that clinically tested HBOCs do not produce coagulopathy at a clinically relevant dose. However, only around 2000 subjects have been tested with multiple HBOCs, and post-marketing data on coagulation has not been published, despite approval of one product in South Africa and Russia for over 10 years (HBOC-201).

## 34.8 Estabishment of Need for Oxygen Delivering Resuscitation Fluids

One of the main areas of interest for application of HBOCs is in the setting of major trauma and hemorrhage. In traumatic hemorrhage, patients may develop an acute coagulopathy which worsens hemorrhage from primary bleeding sites (Hauser et al. 2010). Conventionally, treatment of traumatic coagulopathy involves administration of blood products with a goal of correcting abnormal coagulation test results. However, in major injuries, current blood products can be inadequate to reverse coagulopathy, and HBOCs and other resuscitative fluids may contribute to further dilutional coagulopathy. This leaves room for attempts to develop improved resuscitation fluids that specifically address coagulopathy.

A randomized clinical trial showed that administration of recombinant factor VIIa as an adjunct to direct hemostasis in major trauma reduced the total amount of blood product used (Hauser et al. 2010). However, there was no difference in mortality.

## 34.9 Potential Approaches to Development of Improved Resuscitation Fluids

As research continues in the area of HBOCs, attempts to improve HBOCs as resuscitation fluids specifically in the area of coagulopathy include supplementing the fluids with coagulation factors and comedicating with procoagulants. One study showed that crosslinking fibrinogen to hemoglobin to form polyhemoglobin-fibrinogen resulted in similar clotting times in vitro and in vivo as whole blood in up to 98 % exchange transfusion (Wong and Chan 2007). In contrast, polyhemoglobin alone increased normal clotting time (1–2 min) to greater than 10 min

after 80 % exchange transfusion in a rat model. Another study showed that administration of recombinant factor VIIa improved PT and aPTT after progressive hemodilution had increased clotting time (Darlington et al. 2011).

## 34.10 Discussion and Summary

Several of the studies above do suggest that current HBOCs can contribute to mild platelet dysfunction when used in large quantities (Jahr et al. 2008a, 2008b; Arnaud and Handrigan 2006; Jahr et al. 2010). Part of this is the result of a dilutional coagulopathy that is to be expected with large volume resuscitation with any fluid devoid of clotting factors and platelets. However, thus far, the studies indicate that HBOCs do not produce more coagulopathy and platelet dysfunction when compared to hetastarch fluids. Some of the unique properties of HBOCs, such as the risk of oxidation to methemoglobin, have also been studied with respect to effect on coagulation. In an experimental model (Moallempour et al. 2009), large concentrations of methemoglobin (65 %) did impair clot propagation. As fresh samples of HBOC contain only 1 % methemoglobin, this effect may not be ultimately clinically relevant. Thus far HBOCs do not appear to contribute to more platelet dysfunction than other currently acceptable resuscitation fluids. Nonetheless, as HBOCs continue to be developed and refined, careful consideration of their possible unique effects on platelet function and coagulation is prudent.

Allogeneic human blood for transfusions has significant limitations such as short shelf life of blood products, risk of disease transmission, immunomodulation, risk of hemolytic transfusion reactions and logistical constraints (Chen et al. 2009; Jahr et al. 2011). Unmodified stroma free hemoglobin is not safe as a blood substitute as it causes severe adverse effects: short circulation time in vivo, nephrotoxicity, high affinity to nitric oxide (NO) and related vasoconstriction and hypertension Squires (2002; Reid 2003). Multiple modifications of hemoglobin (Hb) based on intra- and intermolecular cross-linking or encapsulation have been tested to find a viable oxygen carrier. Hemoglobin-based oxygen carriers (HBOCs) have been investigated intensively during the last 30 years with the aim to develop an universal blood substitute. Early generation HBOCs such as diaspirin cross-linked Hb, and later generations, with weakly polymerized Hbs like HBOC-201 and Poly PLP-Hb (PolyHeme®, Northfield Corp.) have prolonged intravascular retention time but caused substantial hypertension Chen et al. 2009; Saxena et al. 1999; Zhang et al. 2011. Sakai et al. and Cabrales et al. (2009) demonstrated that the extent of the vasoconstriction and hypertension decreases with increasing size of the HBOC, (Saxena et al. 1999; Zhang et al. 2011). Additionally although not definitively proven, HBOCs with high p50 may release excessive amounts of oxygen into the systemic circulation also inducing vasoconstriction (Lee et al. 2000). Therefore, polymerization of the Hb leading to larger HBOCs with low p50 (high oxygen affinity) may provide a better solution for prevention of vasoconstriction and can lead to suitable blood substitutes (Winslow 2000; Winslow 2003).

However, regarding coagulation, it is hypothesized that unrelated to the oxygen carrying capacity, larger molecules may worsen coagulation abnormalities (Sakai et al. 2002). Free hemoglobin and tetrameric HBOCs have been studied for over 60 years (Jahr et al. 2011; Reid 2003), with first generation having multiple safety issues (renal toxicity, and death) causing trials to be halted (HemAssist®, Baxter, Hemolink®, Hemosol). Second generation HBOCs were studied in great detail, with a number of large clinical trials published (Jahr et al. 2008b). HBOC-201, (OPK Biotech) has been approved for human use in two countries (South Africa and Russia) without evidence of coagulopathy (Williams et al. 2002). Third generation products, such as Zero-link Hb polymer, also have been studied in pre-clinical models and demonstrate no clinically meaningful coagulation (Jahr et al. 2008b). As the studied compound becomes larger to minimize nitric oxide scavenging and hypertension, so may the likelihood of coagulopathies be worsened.

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# Chapter 35
# Redox Activity of Cell-Free Hemoglobin: Implications for Vascular Oxidative Stress and Endothelial Dysfunction

**Felice D'Agnillo**

## 35.1 Introduction

Hb-based oxygen carriers (HBOCs) have been proposed for a number of applications including resuscitation from hypovolemic shock, elective surgery, ischemia, and others. Given their desirable storage and compatibility characteristics, HBOCs may be particularly useful in military and mass casualty settings such as natural disasters. Early obstacles in the development of HBOCs were largely overcome with the application of a variety of protein modification strategies including intra- and intermolecular crosslinking, conjugation, and polymerization that were primarily aimed at improving the circulation time and oxygen transport properties of these products (Riess 2001; Alayash et al. 2007). This led to the manufacture of several HBOC candidates that generally demonstrated reasonable safety and efficacy in animals and early phase clinical trials (Jahr et al. 2012; Buehler et al. 2010). However, unresolved safety issues highlighted by serious cardiovascular adverse events and death reported for certain HBOCs in Phase III pivotal trials has slowed the development of HBOCs in the United States (Buehler et al. 2010; Silverman et al. 2009). Pre-existing endothelial dysfunction has been cited as an underlying factor in the adverse reactions to HBOCs emphasizing the need to better understand and evaluate the interactions of HBOCs

The findings and conclusions in this chapter have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any agency determination or policy.

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with the vascular system through more predictive preclinical testing (Buehler et al. 2010; Silverman et al. 2009; Biro 2012; Yu et al. 2010). While the mechanisms underlying HBOC-induced vascular dysfunction are likely multi-faceted, the propensity for Hb to generate and propagate oxidative stress has long been proposed as a contributing factor (Alayash et al. 2007; Alayash 1999). This chapter will provide a brief overview of the redox reactions of Hb and how these can lead to highly reactive and cytotoxic protein-bound and unbound species. Recent in vitro and in vivo studies that have focused on the interaction of cell-free and modified Hbs with the vascular endothelium will also be discussed.

## 35.2 Hemoglobin Redox Reactions

### *35.2.1 Auto-Oxidation*

Hb is a tetrameric protein consisting of two alpha and two beta chains each containing a heme prosthetic group. Heme consists of a protoporphyrin IX ring with an iron atom bound to the four nitrogen atoms of the ring and to the imidazole nitrogen of the proximal histidine at position 87 and 92 in the alpha and beta chains, respectively. Ferrous Hb ($HbFe^{2+}$) is capable of sharing an electron allowing for the reversible binding of oxygen. $HbFe^{2+}$ can undergo slow spontaneous auto-oxidation to non-oxygen binding methemoglobin (ferric Hb, $HbFe^{3+}$) (Eq. 1, Table 1). This reaction generates superoxide anion ($O_2^{\bullet -}$), the one electron reduction product of molecular oxygen, which can be converted to hydrogen peroxide ($H_2O_2$) spontaneously or via superoxide dismutase (SOD). Inside the RBC, this auto-oxidation process is controlled by a number of antioxidant enzymes including SOD, catalase, and glutathione peroxidase as well as methemoglobin reducing systems. Outside the RBC, uncontrolled auto-oxidation of cell-free Hb or HBOCs can lead to potentially high amounts of non-functional and less

**Table 1** Redox reactions of hemoglobin

| Redox Mechanism | Reaction Equation |
|---|---|
| Autoxidation | $HbFe^{2+} + O_2 \rightarrow HbFe^{3+} + O_2^{\bullet -}$ **(Eq. 1)** |
| $H_2O_2$-Driven Redox Cycling | $HbFe^{2+} + O_2 + H_2O_2 \rightarrow HbFe^{4+}{=}O + H_2O + O_2$ **(Eq. 2)** |
| | $HbFe^{2+} + H_2O_2 \rightarrow HbFe^{4+}{=}O + H_2O$ **(Eq. 3)** |
| | $HbFe^{3+} + H_2O_2 \rightarrow {}^{\bullet}HbFe^{4+}{=}O + H_2O$ **(Eq. 4)** |
| | $HbFe^{4+}{=}O + H_2O_2 \rightarrow HbFe^{3+} + H_2O + O_2^{\bullet -}$**(Eq. 5)** |
| | ${}^{\bullet}HbFe^{4+}{=}O + H_2O_2 \rightarrow HbFe^{3+} + H_2O + O_2$ **(Eq. 6)** |
| NO-Mediated Oxidation | $HbFe^{2+} + O_2 + NO \rightarrow HbFe^{3+} + NO_3^{\bullet -}$ **(Eq. 7)** |
| Hydroxyl Radical Formation | $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$ **(Eq. 8)** |
| | $O_2^{\bullet -} + Fe^{3+} \rightarrow Fe^{2+} + O_2$ **(Eq. 9)** |
| | $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$**(Eq. 10)** |
| (Net reaction) | $H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + {}^{\bullet}OH + OH^-$**(Eq. 11)** |

stable methemoglobin (Lee et al. 1995; Linberg et al. 1998; Buehler et al. 2007; Faivre et al. 1994; Dunne et al. 2006). Reducing compounds in the plasma such as ascorbate and urate may help limit the auto-oxidation of cell-free Hbs (Buehler et al. 2007; Faivre et al. 1994; Dunne et al. 2006). However endogenous plasma reducing systems may be easily overwhelmed with the infusion of high quantities of HBOCs or under disease settings associated with antioxidant depletion.

### *35.2.2 Redox Reactions with Hydrogen Peroxide*

The reaction of Hb with $H_2O_2$ has received considerable attention (Alayash et al. 2007; Alayash 1999; Rifkind et al. 2004; Reeder 2010; Reeder and Wilson 2005; Giulivi and Davies 1994). Both $HbFe^{2+}$ (oxy and deoxy forms) and $HbFe^{3+}$ can react with $H_2O_2$ to produce ferryl Hb ($HbFe^{4+} = O$) (Eqs. 2–4, Table 1). In the case of $HbFe^{3+}$, this reaction also generates a protein-based radical ($^{\bullet}HbFe^{4+} = O$) that can be detected by electron paramagnetic resonance spectroscopy (Eq. 4, Table 1). Ferryl Hb can decay to $HbFe^{3+}$ through a disproportionation reaction with $HbFe^{2+}$, via auto-reduction, or via its reaction with $H_2O_2$ or other substrates (Eqs. 5 and 6, Table 1). The high redox potential of ferryl Hb ($\sim$1.6 V) indicates its oxidizing capability is close to that of the potent hydroxyl radical ($^{\bullet}OH$, 2.3 V). Both the radical and non-radical forms of ferryl Hb are capable of oxidizing lipids and other biological molecules. $H_2O_2$-driven redox transitions are characterized by specific changes in the spectral properties of Hb (Fig. 35.1a). The redox cycling nature of these reactions is highly dependent on the experimental application of $H_2O_2$. For example, the bolus addition of low or equimolar $H_2O_2$ to $HbFe^{2+}$ produces ferryl Hb very quickly which then converts and accumulates as $HbFe^{3+}$ as the added $H_2O_2$ is rapidly consumed (Fig. 35.1b). In contrast, low continuous fluxes of $H_2O_2$, produced by enzymatic sources such as glucose oxidase, generate ferryl Hb more gradually and sustain the ferryl-ferric cycle since $H_2O_2$ is available to react with $HbFe^{3+}$ (Giulivi and Davies 1994; D'Agnillo and Alayash 2001). In biological systems, this "regenerated" ferryl Hb once again becomes available to propagate oxidative damage to susceptible biomolecules (e.g. lipids). Another consequence of the reaction of Hb with $H_2O_2$ is globin chain destabilization with oxidation of specific amino acids on the $\beta$ chains (Jia et al. 2007). Excessive oxidative damage to Hb results in the release of free iron which can react with $O_2^{\bullet-}$ and $H_2O_2$ to produce hydroxyl radical ($^{\bullet}OH$) via the iron-catalyzed Haber–Weiss reaction or superoxide-driven Fenton reaction (Eqs. 8–11, Table 1) (D'Agnillo and Chang 1998a; Gutteridge 1986). Another important action of $H_2O_2$ is through its role as a substrate for the neutrophil enzyme myeloperoxidase which leads to the production of the highly oxidizing hypochlorous acid (HOCl). The oxidation of Hb by HOCl proceeds through the ferryl redox state culminating with heme destruction and iron release (Maitra et al. 2011, 2012).

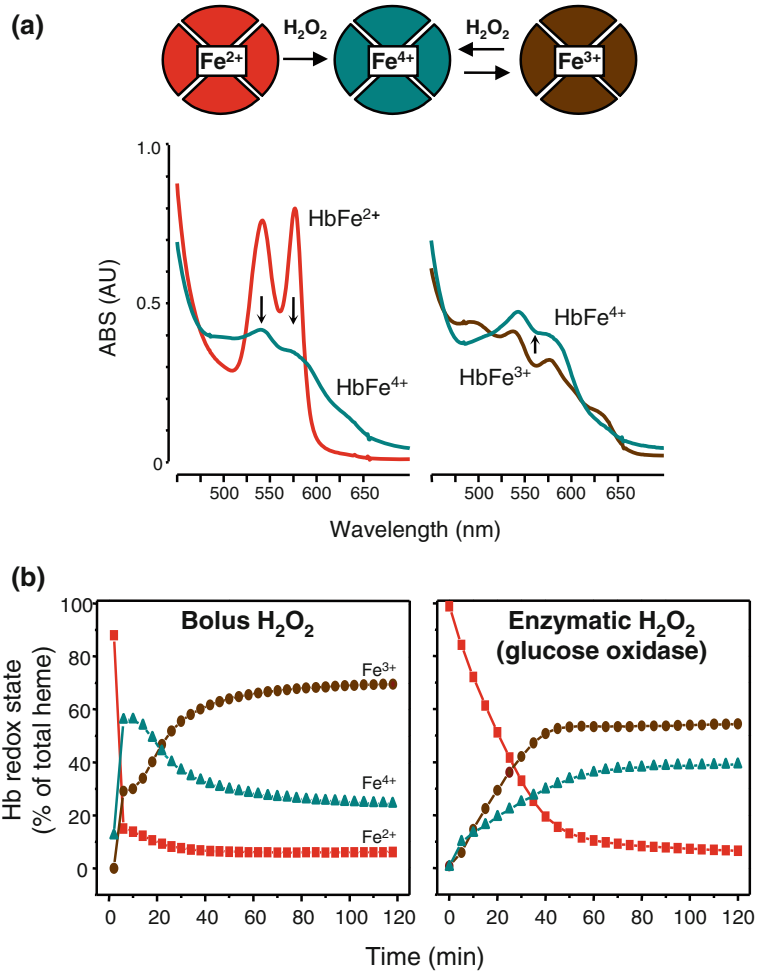


**Fig. 35.1** *Redox cycling of cell-free Hb with hydrogen peroxide*. **a** Schematic representation of the transitions between ferrous ($Fe^{2+}$), ferric ($Fe^{3+}$), and ferryl Hb ($Fe^{4+}$) species induced by hydrogen peroxide ($H_2O_2$). Visible spectra of ferrous Hb, with characteristic peaks at 541 and 577 nm, or ferric Hb, with a peak at 630 nm, undergo $H_2O_2$-induced transition to a spectrum with a characteristic shoulder at 580 nm indicative of ferryl Hb. Positive identification of the ferryl species requires the addition of sodium sulfide to produce a characteristic sulf-Hb compound with a peak at 620 nm (not shown). **b** Hb redox transitions in reaction mixtures containing 50 μM cell-free Hb and bolus addition of $H_2O_2$ (500 μM) or glucose oxidase. At each time point, the composition of ferrous, ferric and ferryl species were calculated by multicomponent analysis. Bolus $H_2O_2$ produces a rapid loss of ferrous Hb with conversion to a short-lived ferryl Hb and the accumulation of ferric Hb as the added $H_2O_2$ is rapidly consumed. In contrast, enzymatic $H_2O_2$ delivery produces a more gradual loss of ferrous Hb in favor of more sustained redox cycling between ferric and ferryl Hb forms

### 35.2.3 Reactions with Nitrogen Species

The reaction of Hb with nitric oxide (NO) is widely considered to be responsible for the vasoactivity of HBOCs observed in preclinical and clinical studies (Alayash et al. 2007; Buehler et al. 2010; Alayash 1999; Cabrales and Friedman 2013). Ferrous Hb (oxy form) reacts very rapidly with NO to generate ferric Hb and nitrate (Eq. 7, Table 1). The reaction of NO with ferric Hb is characterized by the initial rapid formation of a NO-ferric heme adduct followed by a slower process whereby NO reduces heme to the ferrous state forming a NO-ferrous heme complex. NO also reacts rapidly with $O_2^{\bullet -}$ to generate the highly reactive and cytotoxic peroxynitrite ($ONOO^-$) species (Beckman and Koppenol 1996). Peroxynitrite also reacts with $HbFe^{2+}$ via a direct one-electron oxidation to generate ferric Hb though the production of the ferryl species has also been proposed (Alayash et al. 1998; Exner and Herold 2000). Recent studies have proposed the administration of sodium nitrite as a NO supplementation strategy to mitigate the vasoactivity of cell-free Hb (Rodriguez et al. 2009; Minneci et al. 2008; Moon-Massat et al. 2012). This is based on the hypothesis that Hb within the RBC can function as an allosteric nitrite reductase allowing nitrite ($NO_2-$) to react with deoxy-Hb through a series of intermediates to generate NO (Minneci et al. 2008; Basu et al. 2007). However there is some debate as to whether the potential benefits of this NO-generating pathway may be confounded by the reaction of $NO_2-$ with cell-free ferrous Hb (oxy) which generates unstable ferric Hb and nitrate ($NO_3-$) (Moon-Massat et al. 2012; Keszler et al. 2008; Piknova et al. 2009; Buehler et al. 2011).

## 35.3 Endothelial Responses to Cell-Free Hb In Vitro

### 35.3.1 Exposure to Oxidized Hb and Hemin

Vascular endothelial cells play a critical role in the physiological and pathophysiological response to cell-free Hb and HBOCs by virtue of their direct proximity to the circulating blood. Much of our understanding on the potential cytotoxic effects of cell-free Hb on vascular endothelium has been derived from cell culture studies. Purified cell-free Hb or HBOCs in their reduced form ($Fe^{2+}$) are generally not considered cytotoxic to endothelial cell cultures. However, endothelial cells are highly susceptible to cytotoxic effects of heme released by Hb (Balla et al. 2007). Activated leukocytes were shown to promote this effect by oxidizing ferrous Hb to unstable methemoglobin (Balla et al. 1991). Free heme also mediates lipid peroxidation and damage during atherogenesis (Nagy et al. 2010). Oxidized Hb and heme activate NF-$\kappa$B-mediated inflammation responses with increased expression of endothelial surface adhesion molecules ICAM-1, VCAM-1, and E-selectin (Silva et al. 2009; Wagener et al. 1997; Simoni et al. 1997). Heme was recently

shown to mediate endothelial cytotoxicity through lipid peroxidation-induced mitochondrial dysfunction (Higdon et al. 2012). Endothelial exposure to Hb or heme induces cytoprotective heme catabolic (e.g. heme oxygenase) and iron sequestration systems (e.g. ferritin) (Balla et al. 2007; Nagy et al. 2010). Enhanced auto-oxidation of alpha-crosslinked Hb compared to native Hb correlated with a higher increase in endothelial HO-1 (heme oxygenase-1) expression (Motterlini et al. 1995). Interactions of NO with Hb also modulate endothelial HO-1 induction by mechanisms dependent on the redox state of Hb (Foresti et al. 2006). Increased Hb oxidation correlated with increased lipid peroxidation in an endothelial model of hypoxia/reoxygenation (McLeod and Alayash 1999; D'Agnillo et al. 2000). Bovine aortic endothelial cells treated with cell-free Hbs and bolus amounts of $H_2O_2$ showed significant depletion of glutathione (GSH) and cellular necrosis (D'Agnillo et al. 2000; D'Agnillo and Alayash 2000). However the cytotoxicity caused directly by Hb redox activity in these studies was difficult to interpret because the bolus addition of supraphysiological amounts of $H_2O_2$ alone causes significant cytotoxicity.

### *35.3.2 Exposure to Ferryl-Ferric Hb Redox Cycling*

To examine the cellular effects of Hb redox cycling in a more physiologically-relevant setting, the glucose oxidase system was used to produce subtoxic steady state concentrations of $H_2O_2$ ranging from 1 to 10 μM (D'Agnillo and Alayash 2001; D'Agnillo and Alayash 2002). This experimental approach better mimics the steady state $H_2O_2$ levels in human plasma that ranges between 0.1 to 35 μM and may be as high as 100 μM in localized sites of inflammation (Tsukimori et al. 2008; Lacy et al. 1998; Deskur et al. 1998; Halliwell et al. 2000). With this enzymatic $H_2O_2$ delivery system, we reported that ferryl-ferric redox cycling of cell free Hb and alpha-crosslinked Hb induced $G_{2/M}$ cell cycle arrest and apoptotic death in bovine endothelial cell cultures (D'Agnillo and Alayash 2001). Cytotoxicity was reduced by blocking the heme site with cyanide to inhibit Hb redox cycling. The protective effects of catalase (a scavenger of $H_2O_2$) and ascorbate (a known reductant of the ferryl species) as well as the increased susceptibility of GSH-depleted endothelial cells to ferryl cytotoxicity further confirmed the involvement of oxidative injury processes (D'Agnillo and Alayash 2001; D'Agnillo and Alayash 2002). Redox active Hb was also shown to enhance LPS-induced endothelial cytotoxicity using the glucose oxidase system (D'Agnillo 2004).

Recent studies in our laboratory have examined the effects of Hb redox cycling on the oxidative stress response of endothelial cells and specifically on the regulation of antioxidant, detoxifying, and stress proteins that can eliminate protein-bound and unbound species capable of generating oxidative stress. Among several redox-sensitive transcription factors, nuclear factor erythroid 2-related factor 2 (Nrf-2) has emerged as the master regulator of a vast array of detoxifying and

antioxidant proteins including NAD(P)H quinone oxidoreductase-1 (NQO1), GSH biosynthetic enzymes, ferritin, and HO-1 (Ma 2013). Under normal or unstressed conditions, Nrf-2 is mainly sequestered in the cytoplasm by a cluster of proteins that target its degradation via the proteosome. Under oxidative stress, Nrf-2 translocates to the nucleus where it binds gene promoters through specific antioxidant responsive elements (AREs) (Fig. 35.2a). Consistent with a role for the Nrf-2 pathway in the response to Hb oxidative stress, we recently found that redox active Hb induces nuclear translocation of Nrf-2 and promotes the upregulation of HO-1 in primary human lung endothelial cells (Fig. 35.2b). An important potential consequence of endothelial oxidative stress is the loss of barrier function which is often characterized by redox-induced changes in the expression, distribution, or phosphorylation of proteins making up endothelial tight junction (TJs) and adherens junctions (AJs) (Usatyuk and Natarajan 2012; González-Mariscal et al. 2011). Among these proteins, cytoplasmic zona occluden-1 (ZO-1) is a redox-sensitive scaffolding protein that binds the tight junction claudin proteins to the actin cytoskeleton (González-Mariscal et al. 2011). Preliminary studies in our laboratory have shown that redox active Hb may trigger barrier dysfunction as evidenced by the disruption in the cortical distribution pattern of ZO-1, increased central actin stress fibers, and interendothelial gap formation in primary human microvascular endothelial cells (Fig. 35.2c). Understanding the interplay between Hb oxidative stress and Nrf-2 responses in relation to endothelial barrier function is a key area of investigation that should provide important insight on the vascular response to HBOCs.

## 35.4 Hb Redox Activity and Vascular Oxidative Stress In Vivo

### *35.4.1 Indices of Hb Oxidation and Breakdown*

Vascular endothelial dysfunction often accompanies the intravascular hemolysis observed with cerebral hemorrhage, infections, hemodialysis, and hemolytic anemias. Plasma Hb concentrations in these settings are typically in the low micromolar range compared to the millimolar amounts that can be reached with infused HBOCs. These levels can easily exceed the binding capacities of normal plasma (e.g. haptoglobin [Hp]) and cellular receptor systems (e.g. Hb-Hp scavenger receptor CD163). Exposure to cell-free Hb is toxic to the vasculature and major organs including the heart, kidney, lung, liver, and brain (Buehler and D'Agnillo 2010; Buehler et al. 2012; Boretti et al. 2009). The extent of toxicity is mainly linked to the duration of Hb exposure, the state of endogenous antioxidant and detoxification systems, and the potential for aggravation of co-existing pathophysiology such as atherosclerosis and ischemia. These processes are directly influenced by the conversion of circulating or extravasated cell-free Hb to its

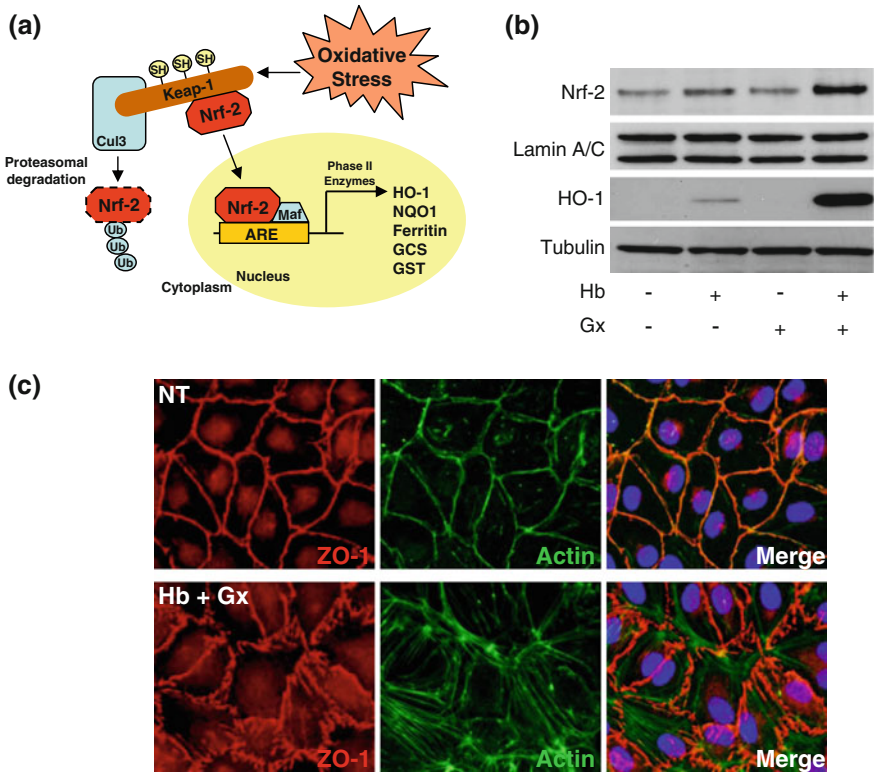


**Fig. 35.2** *Redox active Hb induces endothelial oxidative stress and dysfunction*. **a** Schematic representation of the Nrf-2 pathway. Under normal conditions, Nrf-2 is sequestered in the cytoplasm via binding to its repressor molecule, Keap1, which associates with Cul3, a ubiquitin ligase, that targets Nrf2 for proteasomal degradation. Under conditions of oxidative stress, oxidation/modification of sulfhydryl (SH) groups on KEAP1 leads to dissociation of Nrf-2. Released Nrf-2 translocates to the nucleus where in conjunction with small Maf proteins binds to the antioxidant responsive elements (ARE) in the promoter regions of the target genes for Phase II detoxification enzymes including heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase-1 (NQO1), glutathione transferases (GSTs), $\gamma$-glutamylcysteine synthetase ($\gamma$-GCL), and others. **b** Redox active Hb activates endothelial Nrf-2 and HO-1. Primary human microvascular endothelial cells were treated with medium alone (control), cell-free Hb, glucose oxidase (Gx), or the combination of Hb and Gx for 12 h. Western blot analysis of Nrf-2 and HO-1 in nuclear and whole cell lysates, respectively, showed enhanced nuclear translocation of Nrf-2 and induced HO-1 expression in cells treated with the combination of Hb and Gx. **c** Immunofluorescence of ZO-1 and F-actin in human endothelial cultures. The combination of Hb and Gx induces ZO-1 rearrangement (*red*) along the interendothelial borders and actin stress fibers (*green*). Magnification 400×

oxidation or breakdown products such as methemoglobin, ferryl Hb, and free heme or iron. The measurement of specific markers of Hb oxidative and catabolic processes may therefore be useful in probing mechanisms of toxicity as well as tissue sites of Hb/heme exposure. For the purpose of this discussion, this chapter

will highlight some of our laboratory's findings on markers of Hb oxidation and catabolism measured in rats and guinea pigs exchange transfused with purified cell-free Hbs and polymerized bovine Hb (Oxyglobin®, HbG), a FDA-approved HBOC for veterinary use. However, it should be emphasized that not all HBOCs have the same properties and thus these markers could be differentially expressed depending on differences in molecular size profile, extravasation rate, and susceptibility to oxidation and the release of heme.

Methemoglobin formation has been reported in sheep, rabbits, and humans following large volume HBOC infusion (Lee et al. 1995; Dunne et al. 2006; Sprung et al. 2002). Recent studies in our laboratory showed that polymerized bovine Hb oxidizes more readily in the circulation of guinea pigs, a non-ascorbate-producing species with similar plasma and tissue antioxidant status to humans, compared to rats, an ascorbate-producing species (Buehler et al. 2007). Plasma methemoglobin formation correlated with globin chain destabilization and increased heme release detectable by mass spectrometry (Buehler et al. 2007; Butt et al. 2011). These findings have suggested that the use of animal species with antioxidant capabilities similar to that of humans may be particularly relevant for monitoring the in vivo oxidation of HBOCs.

Heme-to-protein cross-linked Hb species are specifically generated via ferryl redox reactions, and thus have been employed as an in vivo indicator of ferryl-mediated events (Reeder and Wilson 2005; Reeder et al. 2002). Increased levels of heme to protein cross-linked Hb species were found in the cerebrospinal fluid of patients following subarachnoid hemorrhage, suggesting a possible role for ferryl Hb in the delayed vasospasm associated with subarachnoid hemorrhage (Reeder et al. 2002). Increased lipid peroxidation with $F_2$-isoprostane production occurs in animals with glycerol-induced rhabdomyolysis and in patients with rhabdomyolysis (Moore et al. 1998). These effects were caused by lipid peroxide- or $H_2O_2$-driven formation of ferryl Mb in kidneys, and not by free heme or iron-mediated events.

Heme catabolic and iron sequestration processes play an important role in mediating the physiological and pathophysiological responses to HBOCs. Inducible HO-1 and constitutive HO-2 catalyze the degradation of heme to biliverdin (an intermediate in the production of bilirubin), free iron, and carbon monoxide. HO-1 upregulation is often accompanied by ferritin induction as a mechanism to trap liberated iron and limit oxidative stress. Iron overload conditions are typically associated with the conversion of excess tissue ferritin to hemosiderin. Exchange transfusion with polymerized Hb upregulated HO-1 and ferritin expression and increased non-heme iron deposition in kidney, lung, heart, spleen, liver, aorta, and brain (Alayash et al. 2007; Buehler et al. 2010; Buehler and D'Agnillo 2010; Butt et al. 2010, 2011). In the kidney, comparative analyses between rat and guinea pig showed that species differences in antioxidant capabilities could impact the extent to which heme catabolic and iron sequestration systems are activated following HBOC infusion (Butt et al. 2010). In the rat lung, polymerized Hb infusion induced intense HO-1 expression and iron accumulation primarily in alveolar macrophages within the first 12 h post-transfusion. Alterations in lung architecture and extravasation were notable within the first 12 h but resolved by 24 h (Buehler and D'Agnillo 2010).

Others have reported that ferric Hb infusion produced a more intense pulmonary HO-1 response than ferrous Hb and proposed that this may be related to the greater propensity of ferric Hb to release heme (Balla et al. 1995). In the guinea pig heart, polymerized Hb induced significant perivascular activation and accumulation of HO-1 and Iba-1-positive monocytes/macrophages around large vessels and in smaller vessels between myocytes consistent with either free heme or Hb entrance into sub-endothelial spaces (Fig. 35.3). This is particularly important as activated monocytes/macrophages are key sources of reactive oxygen and nitrogen species that can exacerbate the redox activity of cell-free Hb in the intravascular and perivascular compartment. Many regions with high HO-1 expression often colocalized with areas of myocyte degeneration. Increased plasma levels of cardiac troponin I were also noted. Disease states such as myocardial infarction, ischemia–reperfusion, and sepsis are characterized by increased vessel permeability, recruitment of leukocytes, increased oxidant production, and antioxidant depletion (Granger and Korthius 1995). Each of these factors could be aggravated by the presence of cell-free Hb. Preclinical studies with alpha-crosslinked Hb also reported myocardial lesions in rhesus monkeys and swine (Burhop et al. 2004). These lesions were localized to highly vascularized regions of the heart and typically affected <3 % of total cardiac tissue with variable mononuclear cell infiltration. These lesions were believed to involve the reaction of Hb with NO.

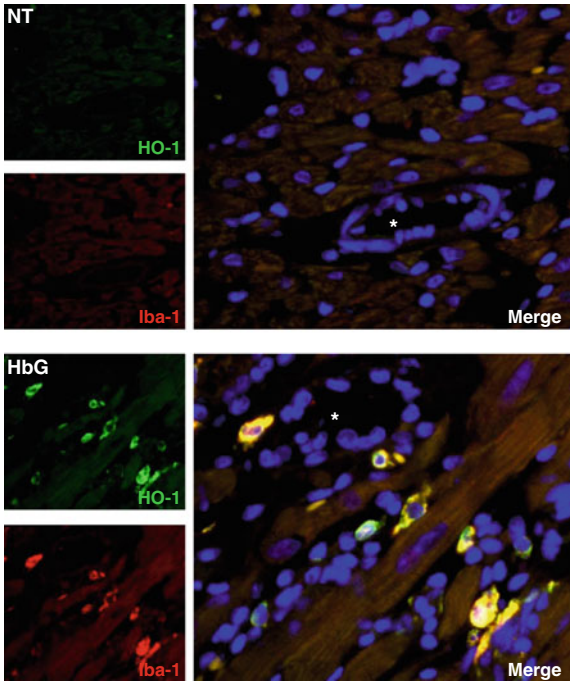


**Fig. 35.3** *Enhanced HO-1 and Iba-1 reactivity in guinea pig heart following infusion with polymerized Hb.* Immunofluorescence staining for HO-1 (*green*) and Iba-1 (*red*), a monocyte/macrophage marker, in the guinea pig heart in sham controls and 24 h after exchange transfusion with polymerized Hb. Nuclear counterstain with Hoechst (*blue*). Asterisk denotes the lumen of a blood vessel. Magnification 400×

### 35.4.2 Indices of Oxidative Stress and Endothelial Barrier Dysfunction

HBOC-induced oxidative stress has the potential to aggravate pre-exisiting endothelial dysfunction associated with hypertension, hypercholesterolemia, diabetes, and septic shock (Buehler et al. 2010; Silverman et al. 2009; Biro 2012; Yu et al. 2010). Endothelial dysfunction is broadly defined as the dysregulation of the vasodilatory, anti-coagulative, and anti-adhesive properties of endothelium. This is commonly characterized by decreased NO bioavailability caused by impaired NO production and/or increased inactivation of NO by reactive oxygen species produced by endothelium and/or infiltrating leukocytes into subendothelial spaces. In the 1990s, lipid peroxidative-type injuries were reported in major organ systems following exchange transfusion of HBOCs in swine and rats (Simoni et al. 1995; Smith et al. 1990). Protection by the iron chelator desferrioxamine against the vasopressor effect of alpha-crosslinked Hb in rabbit hearts indicated the possible involvement of a free iron-mediated mechanism (Motterlini and MacDonald 1993). Increased salicylate hydroxylation products indicative of hydroxyl radical formation were detected in dogs and rats infused with cell-free Hb and polymerized Hbs (Biro et al. 1995; D'Agnillo and Chang 1998b). Hb-mediated oxidative stress and mast cell degranulation have been linked with the increased microvascular permeability of rat mesentery (Baldwin et al. 2003; Baldwin et al. 2004). Protective effects of the antioxidants selenium and GSH against Hb-induced toxicity have also been demonstrated (Simoni et al. 1995; Baldwin et al. 2004). Conjugation of GSH to Hb complexes is being developed as a strategy to reduce Hb redox activity, lipid peroxidation, and inflammation (Simoni et al. 2000). Chemically attaching the antioxidant enzymes SOD and catalase to Hb also showed protective effects compared to polymerized Hb alone in ischemia-reperfusion and brain injury models (D'Agnillo and Chang 1998b; Powanda and Chang 2002).

More recent studies in our laboratory have examined end products of oxidative damage including 4-hydroxy-2-nonenal (4-HNE) protein adducts and 8-hydroxy-2′-deoxyguanosine (8-OHdG) considered to be more reliable and sensitive markers of lipid peroxidation and DNA damage, respectively. Cell-free Hb and polymerized hemoglobin increased 4-HNE protein adducts and 8-OHdG formation in kidney, heart, lung, and brain (Biro 2012; Buehler and D'Agnillo 2010; Boretti et al. 2009; Butt et al. 2011). Increased 4-HNE protein adducts were detected in the vasculature of major organs including the heart and brain following infusion of polymerized Hb (Figs. 35.4 and 35.5a) (Buehler and D'Agnillo 2010; Butt et al. 2011). Our laboratory recently examined the link between the redox activity of Hb and endothelial dysfunction at the level of the blood brain barrier (BBB) (Butt et al. 2011). The BBB is composed of endothelial cells, basal lamina, astrocyte endfoot processes, pericytes, perivascular macrophages, and neurons. An intact BBB limits the entry of potentially neurotoxic cell-free Hb that can trigger harmful oxidative and inflammatory events in the CNS. Endothelial tight junctions (TJs) are specialized

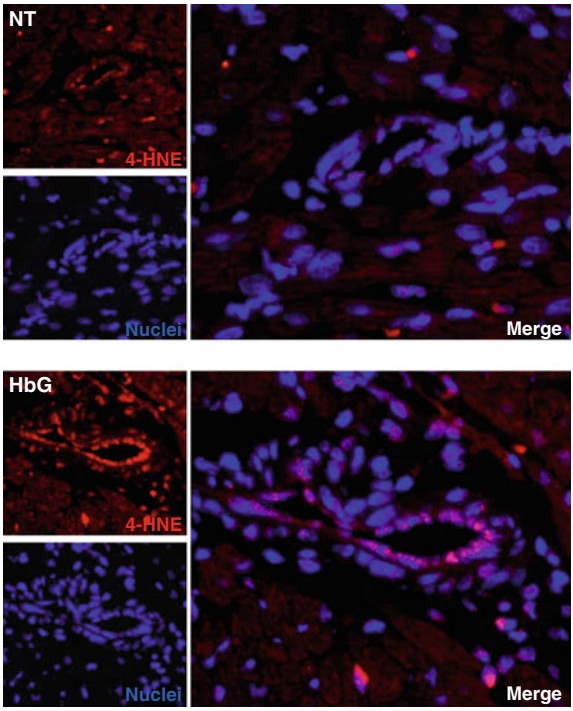


**Fig. 35.4** Vascular oxidative stress in guinea pig heart following infusion with polymerized Hb. Immunofluorescence staining for 4-HNE modified adducts in the guinea pig heart in sham controls and 24 h after exchange transfusion with polymerized Hb (HbG). Nuclear counterstain with Hoechst (*blue*). Magnification 400×

structural complexes that play a critical role in regulating paracellular permeability of the BBB. Our studies have shown that polymerized Hb increases BBB dysfunction as evidenced by altered distribution of the TJ transmembrane protein claudin-5, reduced expression of the TJ scaffolding protein ZO-1, upregulation of astrocyte glial fibrillary acidic protein (GFAP), and IgG extravasation (Butt et al. 2011) (Fig. 35.5b). This was accompanied by increased HO-1 expression in pericytes/perivascular macrophages and infiltrating CD163-positive monocyte/macrophages, but not in microglia, astrocytes, or neurons. Enhanced HO-1 immunoreactivity was observed in blood vessels that showed altered claudin-5 localization and increased GFAP reactivity. Importantly, shed blood- or albumin-transfused control animals did not show TJ protein alterations, increased IgG extravasation, or enhanced HO-1 or GFAP (Fig. 35.5b). Prominent oxidative end product accumulation (4-HNE modified protein adducts and 8-OHdG) along the endothelial lining and immediate perivascular compartments was also noted in vessels that co-expressed HO-1 suggesting that polymerized Hb and/or its breakdown products may preferentially target the endothelial-perivascular cell interface (Butt et al. 2011) (Fig. 35.5a). Oxidative end products and non-heme iron deposition were also variably detected in certain neuron populations. Nitrite-induced oxidation of cell-free Hb was also shown to enhance markers of BBB dysfunction and neurotoxicity (Buehler et al. 2011).

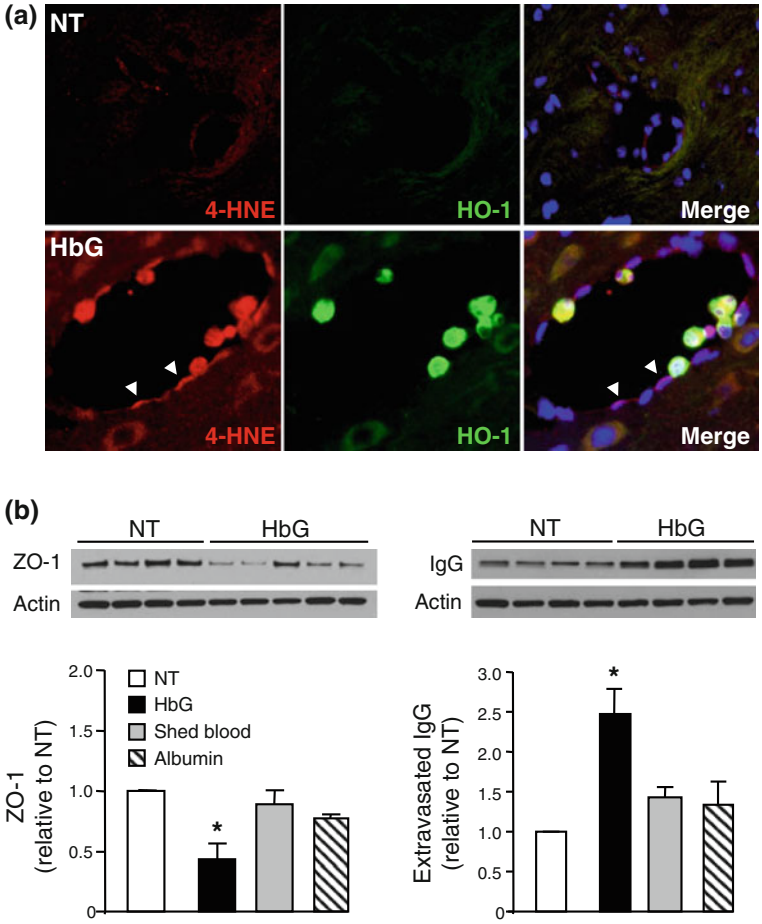


**Fig. 35.5** Vascular oxidative stress and barrier dysfunction in guinea pig brain following infusion with polymerized Hb. **a** Immunofluorescence staining for 4-HNE modified adducts, a marker of lipid peroxidation, and HO-1 in the guinea pig brain 24 h after exchange transfusion with polymerized Hb (HbG). Nuclear counterstain with Hoechst (*blue*). Magnification 400×. **b** Western blot analysis of ZO-1 expression and IgG extravasation after 12 and 72 h, respectively. Representative immunoblots are shown for sham controls and HbG-infused animals (n = 4–5 animals per group). Data from exchange transfusion using shed blood or albumin were included in the densitometry analyses. HbG reduced total ZO-1 expression and increased IgG extravasation while shed blood or albumin-infused animals showed no significant changes in ZO-1 or extravasated IgG levels

## 35.5 Summary

Delineating the mechanisms by which cell-free Hb alters the integrity and function of the vascular system remains an important step toward developing safe and effective HBOCs. Given the similar side effects noted for certain HBOCs it is tempting to speculate the involvement of common mechanisms of toxicity among different HBOCs (Buehler et al. 2010; Silverman et al. 2009). However, this may not be easy to generalize given that not all HBOCs share the same properties in terms of their oxygen affinity, molecular size, ability to extravasate, and susceptibility to oxidize and release heme. Recent evidence continues to strengthen the link between the redox activity of cell-free Hbs and their adverse vascular effects in endothelial cell culture systems and in animal models. This has contributed to the growing recognition that pre-existing endothelial dysfunction and diminished antioxidant defenses may have an important influence on the overall response to HBOCs. Further research using models of endothelial dysfunction and oxidative stress will provide important perspective on the propensity for cell free Hb-induced oxidative events to translate into loss of organ function and toxicity. The insight gained from continued research in this area may help facilitate the rational design of next generation Hb products with reduced pro-oxidant activity and/or promote the use of vascular-directed agents that protect against the oxidative insults of cell-free Hb.

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# Chapter 36
# HBOC Interferences with Routine Clinical Laboratory Tests

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## 36.1 Summary

Volume loading solutions used therapeutically (albumin, dextrans, modified gelatins and hydroxylstarches) are simple plasma substitutes and cannot ensure oxygen transport. The search for blood substitute initially seemed utopian, but this goal is now within our reach, in the form of hemoglobin-based oxygen carriers (HBOC) for example. The clinical development of HBOC has been slowed by adverse effects, including an interference with routine clinical laboratory tests. The understanding of these effects and the possibility of correcting them condition their use on a large scale and the economic consequences which they can generate. To aim of this chapter is to give an overview of recent works related to HBOC interferences with routine clinical laboratory tests.

## 36.2 Brief History of Hemoglobin-Based Oxygen Carriers (HBOCs)

HBOCs have been studied since 1934, when Amberson purified bovine hemoglobin and administered it to study animals, and 1949, when he purified human hemoglobin and infused it into anemic patients (Jahr et al. 2002). The US Army developed a tetrameric alpha-alpha cross-linked hemoglobin (diaspirin cross-linked

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hemoglobin (DCLHb)), which then was produced as the Baxter Corporation product, diaspirin cross-linked hemoglobin (HemeAssist) (Schubert et al. 2003). This product failed clinical trials because of diminished cellular perfusion and increased morbidity and mortality (Schubert et al. 2003). In the mid-1980s, a number of companies developed 'second generation' HBOCs, including Biopure Corporation with Oxyglobin, approved by the Food and Drug Administration (FDA) and the European Union for canine anemia in 1997 and 1998, respectively, and Hemoglobin glutamer-201 (bovine), Hemopure, approved by the South African Drug Council for treatment of human anemia in 2001, and Polymerized pyridoxilated hemoglobin, PolyHeme by Northfield Laboratories (Jahr et al. 2002). To date, there have been a number of studies critically evaluating similar products in animal models (Driessen et al. 2001), and a few clinical trials documenting success in phase 1, 2, and 3 trials (Kasper et al. 1996; Greenburg et al. 2004; Levy et al. 2002). In addition, novel uses of HBOCs, such as evaluation of circulating plasma and blood volume, have been documented (Jahr et al. 2001). Newer strategies are currently being developed with products that alter the earlier generation HBOC characteristics of normal viscosity, lowered hemoglobin (from 10–13 to 4–6 g/dl) and shift of the oxy-hemoglobin curve to the left from the right (p50 30–40 to 6 mmHg) (OxyVita, zero link polymerized HBOC, Oxyvita Incorporated, New York, USA, and MP4, Hemospan, Sangart Incorporated, San Diego, California, USA) (Matheson et al. 2002; Bjorkholm et al. 2005; Smani 2008). These products are in development with published human phase I, II and III studies (Bjorkholm et al. 2005; Olafsson et al. 2006; Olafsson et al. 2008; van der Linden et al. 2011; Olafsson et al. 2011), however, and these strategies for improved efficacy and safety remain to be validated and verified independently.

## 36.3 Physiology of HBOCs

Several classifications of products are under clinical development as either potential oxygen therapeutics and/or volume expanders. The hemoglobin is separated from either the red blood cells or microorganism. If the source of hemoglobin is animal or human, hemoglobin monomers or dimers result. They are associated with a reduced P50, renal infiltration, and a short plasma half-life. To stabilize the small hemoglobin units, they are modified by either cross-linkage, polymerization, or conjugation (Dietz et al. 1996). With cross-linked hemoglobin, the tetrameric structure of hemoglobin is maintained by an intramolecular cross-linking between the alpha-hemoglobin or beta-hemoglobin chains. Polymerized hemoglobin solutions contain either oligomers or cross-linked hemoglobin or polymers of hemoglobin chains (Dietz et al. 1996). Conjugated hemoglobin is formed by linking free hemoglobin to soluble non-hemoglobin polymers. Encapsulated hemoglobin is formed when hemoglobin and enzymes are encapsulated inside artificial red blood cells with artificial membranes, which are either lipid membranes or made of biodegradable material.

## 36.4 Adverse Effects of HBOCs

HBOC solutions have many adverse effects:

*Vasoactivity*: increases in systemic and pulmonary arterial pressure and increases in systemic and pulmonary vascular resistance occur with the use of HBOCs. The underlying mechanism is that hemoglobin, free of red blood cells, leaks through the vascular endothelium and binds to nitric oxide to cause vasospasm. The other mechanism is mediated by the vasoconstrictor peptide, endothelin. Newly discovered allosteric and electric properties of hemoglobin (Jai et al. 1996) seem to control blood pressure and may facilitate tissue oxygenation.

*Nephrotoxicity*: stroma-free hemoglobin resulted in oliguria secondary to acute tubular necrosis. This effect was caused by excessive glomerular filtration of hemoglobin dimers, leading to tubular obstruction. The polymerized or cross-linked HBOCs in current phase III trials do not appear to have this toxicity.

*Interference with macrophage function*: hemoglobin may block the mononuclear phagocytic system and interfere with essential functions such as ingestion of bacteria.

*Antigenicity*: concerns exist with regard to the production of antibodies to xenogeneic hemoglobin.

*Oxidation on storage*: concerns exist with respect to the potential for oxidation during storage and exposure to room air with the resultant production of methemoglobin.

*Activation of complement, kinin, and coagulation*: free hemoglobin increases platelet aggregation by nitric oxide scavenging.

*Iron deposition*: concerns exist regarding hemochromatosis and iron overload.

*Gastrointestinal distress*: clinical findings of abdominal discomfort, pain, nausea, and vomiting have been reported. These findings may be related to nitric oxide binding causing gastrointestinal smooth muscle spasm.

*Neurotoxicity*: stroma-free hemoglobin may cause excitatory neurotoxicity, because free hemoglobin may be neurotoxic. This may be important if the blood–brain barrier is disrupted as in traumatic head injury, subarachnoid hemorrhage, or hemorrhagic stroke. Cole et al. (1997) evaluated the effects of DCLHb in subarachnoid hemorrhage and demonstrated that when injected in the cisterna magna, DCLHb does decrease cerebral blood flow more than an equal volume injection of 'mock cerebrospinal fluid' but less than autologous blood (Cole et al. 1997). In contrast, improved neurologic outcome may be seen in patients with stroke who were administered small DCLHb because DCLHb may enable oxygen delivery, whereas thrombus otherwise hinders the passage of red blood cells (Schubert et al. 2003).

*Free radicals*: it has been demonstrated that stroma free hemoglobin and its breakdown products, heme and free iron, can contribute to the generation of oxygen-free radicals in tissue.

*Interference with diagnosis of transfusion reaction*: this is secondary to hemoglobinuria and the presence of plasma hemoglobin not caused by hemolysis.

One additional area of concern with HBOCs is interference with clinical laboratory values. As HBOC products approach clinical use, it is the responsibility of the laboratory to assess their impact on clinical laboratory values and, along with clinicians, determine what amounts of interference are acceptable for patient care. This chapter sought to give an overview of previous and recent works related to HBOC interferences with routine clinical laboratory tests.

## 36.5 Interference of HBOCs with Clinical Laboratory Tests

The companies now developing HBOCs are altering the hemoglobin molecule chiefly for two purposes: to adjust the molecule's affinity for oxygen and thus achieve effective oxygen transport to tissue and to maintain a large enough molecular size so that the hemoglobin remains in the blood-vascular compartment for a therapeutically sufficient time, and to prevent renal toxicity.

The benefits of new technology never accrue without costs. When compared with donor blood, the potential clinical and economic benefits of blood substitutes are obvious and enormous: increased availability, decreased costs, elimination of infectious agents, no blood types to cross-match, longer shelf-life, etc… (Vlahakes 2000; Dietz et al. 1996). To be able to report valid results, we needed to evaluate the routine laboratory tests used by the intensive care unit (ICU) and to take measures to prevent misinterpretation of the sample appearance by laboratory personnel. Because of its extreme red color, the blood drawn from treated patients appears highly hemolytic. Reports already indicate that HBOCs interfere with many common clinical analyzers, particularly those that require hemoglobin-free plasma or serum (Ali et al. 1997a, b; Balion et al. 1997; Callas et al. 1997; Kazmierczak et al. 1998; Ma et al. 1997; Moreira et al. 1997a, b; Sarkozi et al. 1997; Wolthuis et al. 1999). The interferences from HBOCs seem to be either spectral and/or resulting from chemical interactions with the storage buffer.

Until 2001, it is not known to what extent the various molecular modifications will alter the optical absorbance spectra of the major hemoglobin species and thus affects the spectrophotometric measurements of cooximeters. During their introduction, HBOCs will be a potential source of considerable confusion if all personnel who report or interpret clinical data, from clinicians to medical technologists and laboratorians, are not informed that a patient has received an HBOC. It was reported that a plasma hemoglobin concentration in the grams per deciliter range would ordinarily indicate a severe hemolytic disorder, but large plasma hemoglobin levels are to be expected after the administration of these products. In addition, unexpectedly high readings of carboxyhemoglobin (COHb) or methemoglobin (metHb) could be merely puzzling or cause serious clinical concern, and inexplicably negative COHb values would undermine both the laboratory's and the clinician's confidence in cooximetry results (Ali et al. 2001).

In addition, HBOCs can interfere with pulse oximetry in a dose dependent manner, unrelated to metHb. There is only one published study of the effect which showed at low doses, up to 1.36 g/dl plasma hemoglobin of Hemopure, there was no difference in oxygen saturation as determined by pulse oximetry versus blood gas analysis (Hughes et al. 1996). There is no published information at higher more clinically relevant doses, 5–8 g/dl. From clinical experience, higher levels of PolyHeme of 5–7 g/dl cause variable but dose dependent decreases in pulse oximeter readings despite high arterial $PO_2$ and high saturation by arterial blood gas analysis. Pulse oximeter readings between 78–85 % are common in patients following administration of multiple units of PolyHeme despite adequate oxygenation shown by blood gas. Interference from HBOCs therapy can eliminate pulse oximetry as a useful monitor of oxygenation. Since pulse oximetry is a ubiquitous and often heavily relied upon monitor, its loss not only impacts clinical care but also makes one realize just how much one relies on this monitor. More frequent blood gas analysis is required in patients who have received HBOCs to assess oxygenation particularly in unstable or dynamic situations. Although the data demonstrate convincingly that these HBOCs interfere with the measurements of the test instruments, the next question is whether or not some of the measurements would still be clinically useful. Two encouraging aspects of Ali et al. (2001) study indicate that some of the measurements in the presence of HBOC would be clinically useful. First, they analyzed each material in its undiluted state, whereas in the most frequent anticipated clinical applications, such as blood replacement during surgery, these products would replace only a fraction of the patient's blood volume. Therefore, the results presented in their study represent the worst-case interference that hemoglobin-based blood substitutes cause in the measurements of the test instruments. However, it can also be anticipated that, in desperate attempts to save lives, clinicians will, in some cases of severe hemorrhage, almost completely replace a patient's blood volume with one of these products. The second encouraging aspect of their study is that if clinicians and laboratorians know how a particular HBOC affects the instrument they use, they may be able to extract useful from nonsensical results (Ali et al. 2001).

As indicated previously, HBOCs can interfere with routine clinical laboratory tests including but not limited to serum electrolytes, creatinine, pancreatic and liver enzymes, bilirubin, total protein, prothrombin, partial thromboplastin and lactate (Ali et al. 1997a; Moreira et al. 1997a). The amount of interference is dependent upon the assay being performed, the HBOC, the concentration of HBOC, and the specific laboratory equipment in use. Analyzers utilizing optical/absorbance methods are affected to a greater degree than other types of instruments (Jahr et al. 2002). When HBOCs are used, laboratory personnel should be made aware so that they are able to apply the necessary corrections to ensure accurate results.

The increasing levels of an HBOC (Oxyglobin, Hemopure and Hemolink) in plasma makes the lactate interpretation inaccurate, especially at larger lactate concentrations, causing underestimation of measured lactate values and possible under-treatment of the patient. Therefore, caution must be exercised when

interpreting lactate results when a HBOC is present in plasma (Jahr et al. 2005). Furthermore clinically acceptable results of lactate concentrations were obtained with hemopure with no more than 1.5 g/dl concentration, while hemolink would allow for measurements up to 3 g/dl (Stein et al. 2003). Because cell-free hemoglobin is an avid oxygen scavenger in the absence of reducing enzymes contained within red blood cells, HBOCs exposed to air become rapidly oxidized, increasing the amount of metHb compared with oxyhemoglobin (Yeh and Alayash 2003; Smani et al. 2007). Osgood et al. (2005) have tried to validate the accuracy of lactate measurements using a YSI 2700 SELECT$^{TM}$ Biochemistry Analyzer (YSI Inc, Yellow Springs, OH) in the presence of metHb from oxidized HBOC (metHBOC) and Oxyglobin (Biopure Corp, Cambridge, MA). The correlation between analyzer-measured lactate and calculated or actual lactate concentration was studied. These authors have hypothesized that the presence of metHBOC would interfere with the accuracy of measured lactate values because met-HBOC undergoes a rapid redox reaction with hydrogen peroxide that the YSI 2700 uses, in part, to measure lactate. With less hydrogen peroxide reaching the electrode in the analyzer, a smaller current is generated, and underestimation of actual lactate concentrations could occur (Osgood et al. 2005).

Pancreatic enzymes are also interfered by HBOCs. The levels of amylase and lipase are determined routinely in clinical samples using standard diagnostics that may be limited by the presence of hemoglobin particularly for laboratory assays that use optical methods to quantify parameters if the range of wavelengths utilized are close to that of Hemopure (Biopure Corporation. http://www.hboclab.com/index.php). When Hemopure exceeds a certain concentration in animals this interference may invalidate the results. The degree of interference depends on the individual assay as well as the testing method (Biopure Corporation. http://www.hboclab.com/index.php, Callas et al. 1997; Wolthuis et al. 1999). Untill 3 g/dl of Hemopure in plasma of patients, the analysis of amylase was correct (Wolthuis et al. 1999). In addition, Ma et al. (1997) reported that measurements of amylase and lipase performed on the Hitachi 747 and Vitros 750 were differently affected by bovine polymerized HBOC (Ma et al. 1997). In the same line Arnaud et al. (2011) have demonstrated that amylase measurements on the Vitros 250 are strongly dependent on the level of Hemopure in plasma. Accepting results with a 20 % error above the expected value on this instrument brings the threshold of interference of Hemopure concentrations to around 2.5 g/dl and measurements can be considered accurate on the Vitros 250 for HBOC below this threshold. Clinically, compared to Hextend treatment, Hemopure did seem to elevate the level of amylase and lipase particularly in animals more susceptible to injury, stress or hemorrhagic shock in this study (Arnaud et al. 2011).

Moreover, previous studies have demonstrated that cell free hemoglobin can dramatically interfered with bilirubin measurement in both total and conjugated bilirubin assays (Glick and Ryder 1987; Frank et al. 1978; Shull et al. 1980; Karkoski 1985; van der Woerd-deLange et al. 1983). Ma et al. (1997) have confirmed this effect, regardless of whether the Jendrassik–Grof diazotization method or the Vitros method was used (Ma et al. 1997). Most of the results of

Ma et al. (1997) are consistent with manufacturers' package inserts, stating the limitations of performing analyses on hemolyzed samples, and with samples containing soluble human hemoglobin. In the line of these results, bilirubin-direct test was also influenced by low concentrations of Hemopure (Wolthuis et al. 1999).

It noteworthy to mention that Hemopure at plasma hemoglobin concentrations of 5 g/dl and Hemospan at plasma hemoglobin concentrations of 4 g/dl did not interfere in assays of most "critical care" analytes that might be urgently required peri- or postoperatively (Ma et al. 1997; Cameron et al. 2009). This is not surprising because most electrolyte determinations are performed by ion-selective electrodes and should not be affected by colored substances or by those that scatter light. Ma et al. (1997) also reports electrode-based results from the ABL 505 blood gas analyzer for samples from patients receiving these blood substitutes. No interference was observed in the Vitros 750 enzymatic creatinine method at HBOC concentrations up to 5 g/dl. However, a marked negative interference from HBOC was observed in the Hitachi 747 colorimetric Jaffe method, which measures the picric acid creatinine reaction at 505 and 570 nm. Other "critical care" blood analytes such as glucose and urea were unaffected on one or both of the examined analyzers. It is clear that tests of water and electrolyte homeostasis, acid–base status, renal function, and cardiac damage can be performed for patients receiving these oxygen carriers. In other study, creatine kinase MB-fraction, creatine kinase, gamma-glutamyltransferase, magnesium and uric acid tests were influenced by even low concentrations of Hemopure (Wolthuis et al. 1999). Other HBOC can interfere with alkaline phosphatase assay. PolyHeme, a polymerized hemoglobin, has been shown to interfere with the alkaline phosphatase assay as a result of an absorbance offset caused by alkalin denaturation of hemoglobin (Chance et al. 2000). This interference can be corrected or avoided by modifying the calculation or the analytical wavelength (Chance et al. 2000). Recently, some laboratories have already come up with methods to pretreat serum containing oxygen carriers to prevent their interference in laboratory tests (Sakai et al. 2003).

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# Chapter 37
# Vasoconstriction, Hypertension and Oxidative Toxicity are Regulated by Polymerized Hemoglobin Size

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## 37.1 Negative Side-Effects of Early Generation HBOCs

### *37.1.1 Cell-Free Hb*

Hb was one of the first HBOCs to be evaluated as a RBC substitute (Savitsky et al. 1978). Unfortunately, Hb is able to extravasate through endothelial cell–cell junctions and scavenge NO several orders of magnitude more than RBCs (Suaudeau et al. 1979; Nakai et al. 1998; Dull et al. 2004; Liu et al. 1998; Liao et al. 1999). This is attributed to the smaller molecular dimensions of cell-free Hb compared to the RBC, which allows it to extravasate through the endothelial cell–cell junctions in capillaries into the surrounding tissue space (Suaudeau et al. 1979; Nakai et al. 1998; Dull et al. 2004). Consequently, vasoconstriction (i.e. reduction in blood vessel diameter) ensues, due to scavenging of the gaseous signaling molecule nitric oxide (NO) by the extravasated cell-free Hb (Vogel et al. 1986; Kavdia et al. 2002). Vasoconstriction at the microcirculatory level then leads to systemic hypertension, or high blood pressure (Doherty et al. 1998). In a human clinical trial, human Hb (HbA) induced mild hypertension in patients (Savitsky et al. 1978). HbA has also been shown to elevate the mean arterial blood pressure in hemorrhaged pigs and rats (Hess et al. 1993; Thompson et al. 1994). Therefore in light of these vascular side-effects, Hb is not used as a RBC substitute. Table 37.1 lists the biochemical and biophysical properties of Hb and various HBOCs.

In addition to its' vascular side-effects, cell-free Hb can also elicit tissue toxicity due to its ability to induce oxidative stress. In the blood stream, tetrameric Hb easily dissociates into two $\alpha\beta$ dimers (Alayash 1999; Bunn et al. 1969). The ferrous heme iron of the $\alpha\beta$ dimer autoxidizes to the ferric or methemoglobin

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**Table 37.1** Biochemical and biophysical properties of various HBOCs

| HBOC | MW (kDa) | $P_{50}$ (mm Hg) | n | [Hb] (g/dl) | Viscosity (cP) |
|---|---|---|---|---|---|
| Bovine RBCs | [8 μm] (Rameez and Palmer 2011) | 26.97 (Palmer et al. 2009b) | 2.72 (Palmer et al. 2009b) | N/A | N/A |
| Human RBCs | [8 μm] (Rameez and Palmer 2011) | 32.6 (Gelderman et al. 2010) | 2.23 (Palmer et al. 2009b) | 13 (Napolitano 2009) | 5–10 (Napolitano 2009) |
| bHb | 65.3 (Zhou et al. 2011) | 26.02 (Rameez and Palmer 2011), 26.03 Palmer et al. (2009b), 27.37 (Zhou et al. 2011) | 2.50 Palmer et al. (2009b), 2.75 (Rameez and Palmer 2011), 2.84 (Zhou et al. 2011) | 10 (Zhou et al. 2011) | 1.6 (Zhou et al. 2011) |
| HbA | 62.29 (Zhang et al. 2011) | 13.00 (Rameez and Palmer 2011), 13.27 (Zhang et al. 2011), 13.57 (Palmer et al 2009b) | 2.43 Palmer et al. (2009b), 2.58 (Rameez and Palmer 2011), 2.59 (Zhang et al. 2011) | 10 (Zhang et al. 2011) | 1.6 (Zhang et al. 2011) |
| rHb 1.1 | 64 (Doyle et al. 1999) | 32 (Doherty et al. 1998; Doyle et al. 1999), 33 (Looker et al. 1992) | 2.2 (Doyle et al. 1999), 2.3 (Doherty et al. 1998) | 5 (Stetter et al. 1997),10 (Doyle et al. 1999) | 0.8 (Stetter et al. 1997) |
| DCLHb | 65 = 96 % (Yu et al. 1997)<br>130 = 2–3 % (Yu et al. 1997) | 27.0 (Cashon and Alayash 1995), 32 (Nagababu et al. 2002), 33.9 (Winslow et al. 1998) | 2.07 (Nagababu et al. 2002), 2.4 (Winslow et al. 1998) | 7.9(Winslow et al. 1998) | 1.00 (Winslow et al. 1998) |
| Oxyglobin® | 200 (Rentko et al. 2006; Day 2003) | 34.8 (Alayash et al. 2001), 38.4 (Buehler et al. 2005), 40 (Rentko et al. 2006) | 1.3 (Alayash et al. 2001), 1.4 (Buehler et al. 2005) | 12–14(Rentko et al. 2006) | 1.3 (Rentko et al. 2006) |
| Hemopure® | 250 (Rentko et al. 2006; Day 2003) | 38 (LaMuraglia et al. 2000; Napolitano 2009), 40 (Rentko et al. 2006) | 1.4(Napolitano 2009) | 12–14(Rentko et al. 2006), 13(LaMuraglia et al. 2000; Napolitano 2009) | 1.3(Rentko et al. 2006; LaMuraglia et al. 2000; Napolitano 2009) |
| PolyHeme® | 150 (Day 2003) | 26–32 (Gould et al. 2002; Napolitano 2009), 28–30 (Gould et al. 1998) | 1.7 (Napolitano 2009) | 10(Gould et al. 2002; Napolitano 2009) | 2.1 (Napolitano 2009) |
| Hemolink™ | 32 ≤ 5 % (Adamson and Moore 1998)<br>64 = 33 % (Adamson and Moore 1998)<br>128-600 = 63 % (Adamson and Moore 1998)<br>>600 ≤ 3 % (Adamson and Moore 1998) | 34 (Adamson and Moore 1998), 50.9 (Jia et al. 2004), 52(Nagababu et al. 2002), 52.6(Rohlfs et al. 1998) | 0.97 (Nagababu et al. 2002) 1 (Adamson and Moore 1998), 1.0 (Jia et al. 2004), 1.01 (Rohlfs et al. 1998) | 10 (Adamson and Moore 1998) | [1 cs] (Adamson and Moore 1998) |

(continued)

**Table 37.1** (continued)

| HBOC | MW (kDa) | $P_{50}$ (mm Hg) | n | [Hb] (g/dl) | Viscosity (cP) |
|---|---|---|---|---|---|
| OxyVita™ | $2.5 \times 10^4$ (Bucci et al. 2007), $3.3 \times 10^4$ (Matheson et al. 2002), $4.2 \times 10^4$ (Jia and Alayash 2009) | 3 (Matheson et al. 2002), 6.4(Jia and Alayash 2009; Jahr et al. 2008) | 1.2 (Jia and Alayash 2009; Jahr et al. 2008) | 6 (Jahr et al. 2008) | 2.8 (Jahr et al. 2008) |
| 50:1 T-PolybHb | $1.659 \times 10^4$ (Cabrales et al. 2010; Buehler et al. 2010) | 41 (Buehler et al. 2010) | 0.9 (Cabrales et al. 2010) | 10 (Cabrales et al. 2010; Buehler et al. 2010) | 11.4 (Cabrales et al. 2010; Buehler et al. 2010) |
| 40:1 T-PolybHb | $5.4946 \times 10^4$ (Baek et al. 2012) | 38.1 (Cabrales et al. 2009) | 0.9 (Cabrales et al. 2009) | 10 (Cabrales et al. 2009) | 7.2 (Cabrales et al. 2009) |
| 40:1 R-PolybHb | $2.633 \times 10^4$ (Cabrales et al. 2010; Buehler et al. 2010) | 0.66 (Buehler et al. 2010) | 0.7 (Cabrales et al. 2010) | 10 (Cabrales et al. 2010; Buehler et al. 2010) | 7.8 (Cabrales et al. 2010; Buehler et al. 2010) |
| 30:1 T-PolybHb | $1.3303 \times 10^3$ (Baek et al. 2012; Zhou et al. 2011) | 41.16 (Zhou et al. 2011) | 1.01 (Zhou et al. 2011) | 10 (Zhou et al. 2011) | 14.8 (Zhou et al. 2011) |
| 30:1 R-PolybHb | $6.256 \times 10^3$ (Zhou et al. 2011) | 1.84 (Zhou et al. 2011) | 0.69 (Zhou et al. 2011) | 10 (Zhou et al. 2011) | 9.8 (Zhou et al. 2011) |
| 20:1 T-PolybHb | 749 (Baek et al. 2012; Zhou et al. 2011) | 37.10 (Zhou et al. 2011) | 1.05 (Zhou et al. 2011) | 10 (Zhou et al. 2011) | 4.8 (Zhou et al. 2011) |
| 20:1 R-PolybHb | $1.0259 \times 10^3$ (Zhou et al. 2011) | 2.18 (Zhou et al. 2011) | 0.56 (Zhou et al. 2011) | 10 (Zhou et al. 2011) | 3.6 (Zhou et al. 2011) |
| 10:1 T-PolybHb | 91 (Baek et al. 2012; Zhou et al. 2011) | 29.66 (Zhou et al. 2011) | 1.36 (Zhou et al. 2011) | 10 (Zhou et al. 2011) | 3.2 (Zhou et al. 2011) |
| 10:1 R-PolybHb | 137.950 (Zhou et al. 2011) | 4.54 (Zhou et al. 2011) | 0.61 (Zhou et al. 2011) | 10 (Zhou et al. 2011) | 2.7 (Zhou et al. 2011) |
| 50:1 T-PolyhHb | $1.844 \times 10^4$ (Zhang et al. 2011) | 48.84 (Zhang et al. 2011) | 0.87 (Zhang et al. 2011) | 10 (Zhang et al. 2011) | 9.6 (Zhang et al. 2011) |
| 40:1 T-PolyhHb | $3.68 \times 10^3$ (Zhang et al. 2011) | 37.19 (Zhang et al. 2011) | 0.81 (Zhang et al. 2011) | 9.9 (Zhang et al. 2011) | 8.7 (Zhang et al. 2011) |
| 30:1 R-PolyhHb | $5.54 \times 10^3$ (Zhang et al. 2011) | 0.76 (Zhang et al. 2011) | 0.91 (Zhang et al. 2011) | 9.6 (Zhang et al. 2011) | 6.6 (Zhang et al. 2011) |
| 20:1 R-PolyhHb | $1.10 \times 10^3$ (Zhang et al. 2011) | 1.45 (Zhang et al. 2011) | 0.70 (Zhang et al. 2011) | 9.1 (Zhang et al. 2011) | 3.2 (Zhang et al. 2011) |

**Table 37.1** (continued)

| HBOC | COP (mm Hg) | $k_{off}$, $O_2$ ($s^{-1}$) | $k_{on}$, CO ($\mu M^{-1} s^{-1}$) | $k_{ox}$, NO ($\mu M^{-1} s^{-1}$) | $k_{autox}$($min^{-1}$) |
|---|---|---|---|---|---|
| Bovine RBCs | N/A | 2.01 (Rameez and Palmer 2011) | 0.087 (Rameez and Palmer 2011) | 0.31 (Rameez and Palmer 2011) | N/A |
| Human RBCs | 25 (Napolitano 2009) | 16.67 (Rameez and Palmer 2011) | 0.130 (Rameez and Palmer 2011) | 0.03 (Rameez and Palmer 2011) | N/A |
| bHb | 38 (Zhou et al. 2011) | 36.1 (Buehler et al. 2010; Zhou et al. 2011), 38.97(Rameez and Palmer 2011) | 0.198 (Rameez and Palmer 2011), 0.22 (Buehler et al. 2010; Zhou et al. 2011) | 18.3 (Buehler et al. 2010; Zhou et al. 2011), 28.2 (Rameez and Palmer 2011) | $1.5 \times 10^{-4}$ (Baek et al. 2012) |
| HbA | 38 (Zhang et al. 2011) | 38.71 (Rameez and Palmer 2011), 40.4(Zhang et al. 2011) | 0.200 (Rameez and Palmer 2011), 0.214 (Zhang et al. 2011) | 18.5 (Zhang et al. 2011), 27.5 (Rameez and Palmer 2011) | $5.54 \times 10^{-4}$ (Zhang et al. 2011) |
| rHb 1.1 | 42 (Doyle et al. 1999) | N/A | N/A | 58 (Doherty et al. 1998) | $8.5 \times 10^{-4}$ (Doherty et al. 1998) |
| DCLHb | 23.0 (Winslow et al. 1998) | 56.0 (Cashon and Alayash 1995) | 0.17 (Cashon and Alayash 1995) | 31 (Rohlfs et al. 1998) | $3.7 \times 10^{-3}$ (Winslow et al. 1998), $5.5 \times 10^{-3}$ (Cashon and Alayash 1995), $4.00 \times 10^{-4}$ (Nagababu et al. 2002) |
| Oxyglobin® | 42 (Rentko et al. 2006) | 50.9 (Alayash et al. 2001), 60.0(Buehler et al. 2005) | 0.15 (Alayash et al. 2001; Buehler et al. 2005), 0.27 (Sakai et al. 2008) | 66 (Sakai et al. 2008) | $7.8 \times 10^{-3}$ (Buehler et al. 2005), $5.98 \times 10^{-4}$ (Nagababu et al. 2002) |
| Hemopure® | 17 (LaMuraglia et al. 2000), 25(Rentko et al. 2006; Napolitano 2009) | N/A | N/A | N/A | N/A |
| PolyHeme® | 23 (Napolitano 2009) | N/A | N/A | N/A | N/A |
| Hemolink™ | 24 (Adamson and Moore 1998) | 130 (Jia et al. 2004) | 0.12 (Jia et al. 2004) | 31 (Rohlfs et al. 1998) | $6.53 \times 10^{-4}$ (Nagababu et al. 2002) |
| OxyVita™ | 2.2 (Jahr et al. 2008) | 27.4 (Jia and Alayash 2009) | 0.28 (Jia and Alayash 2009) | 30 (Jia and Alayash 2009) | $3.0 \times 10^{-4}$ (Jia and Alayash 2009) |
| 50:1 T-PolybHb | 1 (Cabrales et al. 2010; Buehler et al. 2010) | 53.0 (Buehler et al. 2010) | 0.18 (Buehler et al. 2010) | 18.9 (Buehler et al. 2010) | $1.23 \times 10^{-3}$ (Buehler et al. 2010) |
| 40:1 T-PolybHb | 5 (Cabrales et al. 2009) | N/A | N/A | NA | N/A |

(continued)

**Table 37.1** (continued)

| HBOC | COP (mm Hg) | $k_{off}$, $O_2$ ($s^{-1}$) | $k_{on}$, CO ($\mu M^{-1} s^{-1}$) | $k_{ox}$, NO ($\mu M^{-1} s^{-1}$) | $k_{autox}$($min^{-1}$) |
|---|---|---|---|---|---|
| 40:1 R-PolybHb | 7 (Cabrales et al. 2010; Buehler et al. 2010) | 22.0 (Buehler et al. 2010) | 4.84 (Buehler et al. 2010) | 17.5 (Buehler et al. 2010) | $9.0 \times 10^{-4}$ (Buehler et al. 2010) |
| 30:1 T-PolybHb | 2 (Zhou et al. 2011) | 57.1 (Zhou et al. 2011) | 0.157 (Zhou et al. 2011) | 18.7 (Zhou et al. 2011) | $1.5 \times 10^{-4}$ (Baek et al. 2012) |
| 30:1 R-PolybHb | 14 (Zhou et al. 2011) | 24.7 (Zhou et al. 2011) | 5.95 (Zhou et al. 2011) | 17.5 (Zhou et al. 2011) | N/A |
| 20:1 T-PolybHb | 24 (Zhou et al. 2011) | 58.6 (Zhou et al. 2011) | 0.183 (Zhou et al. 2011) | 17.1 (Zhou et al. 2011) | $1.3 \times 10^{-4}$ (Baek et al. 2012) |
| 20:1 R-PolybHb | 39 (Zhou et al. 2011) | 29.7 (Zhou et al. 2011) | 3.92 (Zhou et al. 2011) | 16.4 (Zhou et al. 2011) | N/A |
| 10:1 T-PolybHb | 42 (Zhou et al. 2011) | 47.2 (Zhou et al. 2011) | 0.193 (Zhou et al. 2011) | 18.9 (Zhou et al. 2011) | $1.2 \times 10^{-4}$ (Baek et al. 2012) |
| 10:1 R-PolybHb | 48 (Zhou et al. 2011) | 29.1 (Zhou et al. 2011) | 6.138 (Zhou et al. 2011) | 17.4 (Zhou et al. 2011) | N/A |
| 50:1 T-PolyhHb | 4 (Zhang et al. 2011) | 51.7 (Zhang et al. 2011) | 0.184 (Zhang et al. 2011) | 20.1 (Zhang et al. 2011) | $1.34 \times 10^{-3}$ (Zhang et al. 2011) |
| 40:1 T-PolyhHb | 4 (Zhang et al. 2011) | 49.7 (Zhang et al. 2011) | 0.181 (Zhang et al. 2011) | 19 (Zhang et al. 2011) | $1.45 \times 10^{-3}$ (Zhang et al. 2011) |
| 30:1 R-PolyhHb | 24 (Zhang et al. 2011) | 25.5 (Zhang et al. 2011) | 4.88 (Zhang et al. 2011) | 20.2 (Zhang et al. 2011) | $6.86 \times 10^{-4}$ (Zhang et al. 2011) |
| 20:1 R-PolyhHb | 37 (Zhang et al. 2011) | 31.4 (Zhang et al. 2011) | 4.76 (Zhang et al. 2011) | 21.8 (Zhang et al. 2011) | $4.98 \times 10^{-4}$ (Zhang et al. 2011) |

(metHb) form at a higher rate than the ferrous heme of tetrameric Hb (Zhang et al. 1991; D'Agnillo 2006). MetHb formation via autoxidation produces the reactive oxygen species (ROS) superoxide anion and indirectly hydrogen peroxide ($H_2O_2$) (D'Agnillo 2006; Misra and Fridovich 1972). Hence, the ROS hydroxyl radical can be generated by the reaction of superoxide with $H_2O_2$, and this is catalyzed by iron released from degraded heme (D'Agnillo 2006; Graf et al. 1984). Hence, Hb oxidation drives the production of ROS (D'Agnillo 2006; Misra and Fridovich 1972; Graf et al. 1984). Unfortunately, in vivo exposure to hydroxyl radicals has been linked to renal failure and apoptosis of vascular cells (Walker and Shah 1988; Shah and Walker 1988; Li et al. 1997). To compound matters further, the small size of $\alpha\beta$ dimers also facilitates their filtration through the kidneys, and thus increases the opportunity for tissue exposure to ROS (Bunn et al. 1969). In clinical human trials, cell-free HbA infusion caused noticeable hemoglobinuria, indicative of HbA clearance through the kidneys (Savitsky et al. 1978). The highly reactive species ferryl Hb can also be formed from further reactions of Hb with $H_2O_2$ (D'Agnillo 2006; Giulivi and Davies 1990; Kanner et al. 1988). Ferrous Hb can be oxidized to ferric Hb via $H_2O_2$ exposure, and ferryl Hb is formed as an intermediate during this reaction (D'Agnillo 2006; Giulivi and Davies 1990). MetHb itself can also be converted to ferryl Hb in the presence of $H_2O_2$ (D'Agnillo 2006; Kanner et al. 1988). Ferryl Hb can elicit inflammation of vascular endothelial cells (Silva et al. 2009). Overall, oxidative stress facilitated by Hb oxidation products is a problem many HBOCs face and aim to prevent.

### *37.1.2 Recombinant HbA 1.1 (rHb 1.1)*

Therefore in order to reduce renal filtration of $\alpha\beta$ dimers, recombinant HbA (rHb 1.1) was developed by Somatogen Inc. (Boulder, CO) (Looker et al. 1992). Mutations to the native structure of HbA include an oxygen affinity lowering asparagine to lysine mutation at the $\beta$-108 position, and the insertion of a glycerin residue which covalently links the neighboring $\alpha$ chains together (Looker et al. 1992; Moo-Penn et al. 1978). Linking the $\alpha$ globin subunits together stabilized the structure of rHb 1.1 and inhibited dissociation of the tetramer into $\alpha\beta$ dimers (Looker et al. 1992). Several clinical human trials demonstrated that prevention of rHb 1.1 dissociation into $\alpha\beta$ dimers led to reduced oxidative stress and diminished renal toxicity (Viele et al. 1997; Hayes et al. 2001). Unfortunately, rHb 1.1 also elicited noticeable levels of hypertension in surgery patients (Hayes et al. 2001). In another clinical trial, rHb 1.1 caused lower esophageal sphincter tension in humans (Murray et al. 1995). It was hypothesized that the mechanism of this inhibition is linked to NO scavenging by rHb 1.1 (Murray et al. 1995). Therefore, although rHb 1.1 exhibited reduced oxidative stress and renal toxicity, it still exhibited undesirable vascular side-effects (Viele et al. 1997; Hayes et al. 2001; Murray et al. 1995). In light of these side-effects and after acquiring Somatogen Inc. (Boulder, CO), Baxter International Inc. (Deerfield, IL) subsequently engineered rHb 3011

with a reduced dioxygenation rate constant in order to eliminate vasoconstriction and systemic hypertension (Olson et al. 2004; Varnado et al. 2012; Baxter 2012). However, it has recently been shown that rHb 3011 autoxidizes at a greater rate in vitro compared to the earlier generation rHb 0.1, which could lead to increased oxidative stress on the vasculature (Varnado et al. 2012).

### *37.1.3 Diaspirin Cross-Linked HbA (DCLHb)*

Site specifically cross-linking HbA represents another strategy to eliminate HbA dissociation into $\alpha\beta$ dimers. Hence, diaspirin cross-linked HbA (DCLHb) was developed by the United States (US) Army and commercially as HemAssit™ by Baxter International Inc. (Deerfield, IL) (Baxter 2012; Winslow 2003). DCLHb is composed of HbA, in which the neighboring $\alpha$ globin chains are covalently cross-linked using bis-(3,5-dibromosalicyl) fumarate (Chatterjee et al. 1986). DCLHb consists of 96 % tetrameric HbA and 2–3 % of HbA tetrameric dimers (Yu et al. 1997). In Phase III clinical trials, DCLHb induced hypertension in stroke patients (Saxena et al. 1999). Elevated blood pressure levels were also reported when DCLHb was infused in patients after cardiac surgery (Lamy et al. 2000). When administered to traumatic hemorrhagic shock patients, DCLHb recipients died more often than saline recipients (Sloan et al. 1999). Despite stabilization of DCLHb's tetrameric structure via $\alpha$ globin chain cross-links, DCLHb readily oxidized in vitro when $H_2O_2$ was present (Cashon and Alayash 1995). Therefore, DCLHb exhibited many of the side-effects associated with transfusion of cell-free Hb and is no longer being pursued by Baxter International Inc. (Winslow 2003; Saxena et al. 1999; Lamy et al. 2000; Sloan et al. 1999; Cashon and Alayash 1995).

## 37.2 Hb Polymerization as a Strategy to Mitigate Vascular Side-Effects

In order to prevent many of the side-effects commonly associated with early commercial HBOCs, the focus has turned to developing HBOCs which are too large to pass through the endothelial cell–cell junctions of blood vessels (Alayash 2004; Palmer 2006). The rationale behind this approach centers on reducing HBOC extravasation into the tissue space so that the HBOC is not in close proximity to the endothelium (Alayash 2004; Palmer 2006). This should lead to less scavenging of endothelial-derived NO by the HBOC, reduced vasoconstriction and hypertension, as well as reduced oxidative tissue toxicity (Alayash 2004; Palmer 2006). In light of this approach, several companies have developed polymerized Hb (PolyHb) solutions as potential RBC substitutes.

### 37.2.1 Oxyglobin®

OPK Biotech LLC (Cambridge, MA) developed two HBOCs, which have gone through extensive studies, namely Oxyglobin® and Hemopure® (OPK Biotech 2012). Both Oxyglobin® and Hemopure® consist of glutaraldehyde polymerized bovine Hb (PolybHb) (OPK Biotech 2012). Glutaraldehyde forms intramolecular cross-links within the Hb tetramer and intermolecular cross-links between neighboring Hb tetramers (Chang 1998). Oxyglobin® is composed of PolybHb with an average MW of 200 kDa (Rentko et al. 2006; Day 2003). It is approved for veterinary use in the US (Day 2003). However, Oxyglobin® has been shown to elicit both vasoconstriction and hypertension in vivo (Tsai et al. 2006). Because of its availability, multiple studies have explored the toxicity of Oxyglobin® Butt et al. (2010, 2011). The oxidative stress caused by Oxyglobin® damaged blood brain barrier endothelial cells and elicited cellular apoptosis in vivo (Butt et al. 2011). Iron was found to be deposited in endothelial cells and neurons associated with the blood brain barrier after administration of Oxyglobin® (Butt et al. 2011) In addition, Oxyglobin® administered to guinea pigs and rats has been shown to facilitate iron deposition in renal tissues (Butt et al. 2010). These results indicate extravasation of Oxyglobin® through endothelial cell–cell junctions and its deposition in the tissue space Butt et al. (2010, 2011).

### 37.2.2 Hemopure®

Hemopure® is a glutaraldehyde PolybHb solution with an average MW of 250 kDa, which was also developed by OPK Biotech LLC (Cambridge, MA) (OPK Biotech 2012; Rentko et al. 2006; Day 2003). Hemopure® is composed of 2 % unpolymerized bovine Hb (bHb) compared to Oxyglobin®, which is composed of 31 % unpolymerized bHb (Rice et al. 2008). A study of resuscitated hemorrhagic shock-induced swine reported that Hemopure® elevated blood pressure less than Oxyglobin®, and this was attributed to the reduced amount of unpolymerized bHb present in Hemopure® compared to Oxyglobin® (Rice et al. 2008). When administered before, during, and after elective aortic surgery, Hemopure® produced hypertension in patients (Kasper et al. 1996; LaMuraglia et al. 2000). Data from another Hemopure® clinical trial highlighted a possible vulnerability in elderly orthopedic surgery patients to negative vascular side-effects (Jahr et al. 2008; Freilich et al. 2009). These findings indicate that despite having less unpolymerized bHb than Oxyglobin®, Hemopure® still presents risks to the vasculature in clinical settings (Kasper et al. 1996; LaMuraglia et al. 2000; Jahr et al. 2008; Freilich et al. 2009; Rice et al. 2008).

### 37.2.3 Polyheme®

Northfield Laboratories Inc. (Evanston, IL) developed a glutaraldehyde polymerized pyridoxal phosphate cross-linked HbA product known as PolyHeme® (Day 2003; Sehgal et al. 1984). PolyHeme® has an average MW of 150 kDa (Day 2003). The pyridoxilated HbA reduces the oxygen affinity of PolyHeme® Sehgal et al. (1981, 1984). In clinical trials, PolyHeme® was administered to trauma, surgery, and hemorrhagic shock patients, and did not increase blood pressure to unsafe levels (Gould et al. 1998, 2002; Moore et al. 2009). Unfortunately, PolyHeme® has been linked to negative side-effects in various animal studies (Yu et al. 2010; Handrigan et al. 2005). A recent study showed that PolyHeme® induced vasoconstriction in lambs (Yu et al. 2010). PolyHeme® administration also produced organ failure and death in hemorrhaged rats (Handrigan et al. 2005). Negative vascular responses are not surprising considering that Oxyglobin® and Hemopure®, both two larger sized glutaraldehyde PolybHbs, displayed similar negative side-effects in vivo (Rentko et al. 2006; Day 2003; Tsai et al. 2006; Kasper et al. 1996; LaMuraglia et al. 2000; Jahr et al. 2008; Freilich et al. 2009; Yu et al. 2010; Handrigan et al. 2005). PolyHeme® production was shut down after ethical questions were raised over the consent requirements of the final clinical trial (Moore et al. 2009; Chen et al. 2009; Kipnis et al. 2006).

### 37.2.4 Hemolink™

Hemosol Inc. (Toronto, Canada) developed the *O*-raffinose cross-linked HbA product Hemolink™ (Day 2003; Adamson and Moore 1998). Hemolink™ has a wide range of MWs which consists of less than or equal to 5 % 32 kDa, 33 % 64 kDa, 63 % 128–600 kDa, and less than or equal to 3 % greater than 600 kDa species (Adamson and Moore 1998). In Phase II and Phase III clinical trials, Hemolink™ administration lead to hypertension in coronary artery surgery patients (Cheng et al. 2004; Greenburg and Kim 2004; Hill et al. 2002). Hemolink™ exhibited diminished renal toxicity in vivo and NO reactions in vitro compared to HbA (Lieberthal et al. 1999). In this study, Hemolink™ also induced hypertension in rats (Lieberthal et al. 1999). Thus despite Hemolink™ having a low affinity for NO in vitro, there was still evidence of systemic hypertension (Lieberthal et al. 1999). Hemolink™ production was discontinued due to negative side-effects observed in clinical trials (Chen et al. 2009).

### 37.2.5 OxyVita™

Of all the aforementioned PolyHbs which have reported average MWs, all of them ranged between 150 and 250 kDa (Rentko et al. 2006; Day 2003). All four of the PolyHbs exhibited some form of vasoconstriction, hypertension, and/or oxidative

stress (Tsai et al. 2006; Butt et al. 2010, 2011; Kasper et al. 1996; LaMuraglia et al. 2000; Jahr et al. 2008; Freilich et al. 2009; Yu et al. 2010; Handrigan et al. 2005; Cheng et al. 2004; Greenburg and Kim 2004; Hill et al. 2002; Lieberthal et al. 1999). The presence of vasoconstriction and systemic hypertension make it reasonable to assume that these commercial HBOCs are still able to extravasate through the endothelium and scavenge NO despite their larger size compared to HbA (Rentko et al. 2006; Day 2003; Adamson and Moore 1998; Tsai et al. 2006; Kasper et al. 1996; LaMuraglia et al. 2000; Jahr et al. 2008; Freilich et al. 2009; Yu et al. 2010; Handrigan et al. 2005; Cheng et al. 2004; Greenburg and Kim 2004; Hill et al. 2002; Lieberthal et al. 1999). Therefore, it is possible that since Oxyglobin® induces oxidative stress, similarly sized HBOCs may possess similar potential for oxidation Butt et al. (2010, 2011). To avoid vasoconstriction, hypertension, and oxidative stress, some groups have developed ultrahigh MW PolyHbs.

OXYVITA Inc. (New Windsor, NY) developed a high MW RBC substitute known as OxyVita™ (OXYVITA Inc. 2012; Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009). Also known as zero-link polymerized bHb, the ultrahigh MW of Oxyvita™ has been reported to be 25, 33 and 42 MDa in separate studies (Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009). OxyVita™ is synthesized by cross-linking the $\beta$ globin chains of bHb with bis(3,5-dibromosalicy)-adipoate (Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009; Kwansa et al. 2000). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide is then used to polymerize the bHb tetramers (Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009). OxyVita™ has been shown to produce low levels of vasoconstriction, which did not lead to hypertension in animal studies (Matheson et al. 2002; Bucci et al. 2007). These benefits have been attributed to the large size of the PolybHb, which prevents its extravasation into the tissue space and thus its inability to scavenge NO (Matheson et al. 2002; Bucci et al. 2007). Therefore, the zero-link cross-linking process appears to be able to prevent vascular side-effects common to many commercial HBOCs (Matheson et al. 2002; Bucci et al. 2007). Despite this major advantage, OxyVita™ possesses a high oxygen affinity (Matheson et al. 2002; Jia and Alayash 2009). The high oxygen affinity makes it unclear how well OxyVita™ will deliver oxygen under physiological conditions compared to other commercial HBOCs (Matheson et al. 2002; Jia and Alayash 2009). OXYVITA Inc. is presently raising resources to pursue further trials of OxyVita™ (OXYVITA Inc. 2012).

### *37.2.6 Overview of Commercial PolyHbs*

Many of the commercial PolyHbs discussed above failed Phase III clinical trials, before their potential for negative side-effects were fully understood (Jahr et al. 2008; Freilich et al. 2009; Moore et al. 2009; Greenburg and Kim 2004; Chen et al. 2009). It is reasonable to expect that if a systematic study of these PolyHbs had been performed they would not have progressed as far without serious design

alterations. Investigations into the gaseous ligand binding/release kinetics, ability to induce vasoconstriction, systemic hypertension, and oxidative stress-inducing potential of these PolyHbs could have foreshadowed the side-effects observed during Phase III clinical trials (Jahr et al. 2008; Freilich et al. 2009; Greenburg and Kim 2004). In the future, PolyHb development should include systematic preliminary studies to prevent a reoccurrence of these common side-effects. Therefore, the wide difference in MW between the low MW PolyHbs Oxyglobin®, Hemopure®, PolyHeme®, Hemolink™ and the ultrahigh MW PolyHb OxyVita™ highlight the need for a systematic study to investigate the effects of PolyHb MW on its' safety profile (i.e. ability to induce vasoconstriction, systemic hypertension and oxidative toxicity) (Rentko et al. 2006; Day 2003; Adamson and Moore 1998; Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009).

## 37.3 Systematic Study of PolyHb MW on Safety Profile

In a series of publications, Palmer's group reported the results of a systematic study investigating the biophysical properties and in vivo responses of variable MW glutaraldehyde PolybHbs (Cabrales et al. 2009; Cabrales et al. 2010; Baek et al. 2012; Palmer et al. 2009a; Buehler et al. 2010; Zhou et al. 2011).

Palmer's group first demonstrated that the quaternary state of bHb could be used to control the oxygen affinity of the resultant PolybHb solution Palmer et al. (2009a). This work took advantage of the fact that Hb can be held in either the tense (T) or relaxed (R) quaternary state by simply fully deoxygenating or fully oxygenating the bHb solution, respectively (Palmer et al. 2009a). Hence, T-state and R-state PolybHbs were synthesized by polymerizing T-state and R-state bHb with glutaraldehyde, respectively (Palmer et al. 2009a). In this work, T- and R-state PolybHbs were synthesized at the following glutaraldehyde to bHb molar ratios (i.e. cross-link densities): 10:1, 20:1, 30:1 and 40:1 (Palmer et al. 2009a). It was observed that T-state PolybHb displayed a higher $P_{50}$ compared to R-state PolybHb (Palmer et al. 2009a). In addition, the cooperativity coefficient of both T- and R-state PolybHb decreased with increasing cross-link density (Palmer et al. 2009a). This result is consistent with increasing cross-linking density, thereby reducing the mobility of the globin chains in the Hb tetramer (Palmer et al. 2009a).

A subsequent study evaluated the effect of T-state PolybHb MW on the extent of vasoconstriction and systemic hypertension (Cabrales et al. 2009). In this study, T-state PolybHbs with glutaraldehyde to bHb molar ratios of 20:1, 30:1, 40:1, and 50:1 were synthesized in the lab (Cabrales et al. 2009). Ultrafiltration was then used to fractionate each PolybHb solution into a fraction greater than 500 kDa in MW and a fraction less than 500 kDa in MW (Cabrales et al. 2009). All of the PolybHb solutions with MW less than 500 kDa were shown to induce vasoconstriction and hypertension in top-loaded hamsters outfitted with the window chamber, and this was likely due to extravasation of low MW PolyHb through the endothelium and subsequent PolybHb scavenging of endothelium-derived NO

(Cabrales et al. 2009). Negative vascular effects were less prevalent for the PolybHbs with MW greater than 500 kDa. (Cabrales et al. 2009). Most notably, no vasoconstriction and only minor hypertension were present with the 40:1 and 50:1 PolybHbs (Cabrales et al. 2009). The diminished vascular side-effects exhibited by the PolybHbs greater than 500 kDa were similar to those observed for the ultra-high MW PolybHb OxyVita$^{TM}$ (Matheson et al. 2002; Bucci et al. 2007; Jia and Alayash 2009; Cabrales et al. 2009). An important distinction between OxyVita$^{TM}$ and the high MW T-state PolybHbs is the high $P_{50}$ of the T-state PolybHbs and the low $P_{50}$ of OxyVita$^{TM}$ (Matheson et al. 2002; Jia and Alayash 2009; Cabrales et al. 2009). Previous work has shown that high $P_{50}$ HBOCs deliver oxygen to tissues more readily versus low $P_{50}$ HBOCs (Sakai et al. 2005).

Therefore, the ability of T-state and R-state PolybHbs to deliver $O_2$ was evaluated in vivo to better understand the effect of PolybHb oxygen affinity in influencing tissue oxygenation (Cabrales et al. 2010). Hence, 40:1 R-state and 50:1 T-state PolybHbs were used in this study, and all HBOCs possessed MWs greater than 500 kDa (Cabrales et al. 2010). It was observed that T-state PolybHb delivered more $O_2$ to tissues in the hamster chamber window model versus R-state PolybHb (Cabrales et al. 2010). As expected, this was largely attributed to the high oxygen affinity of R-state PolybHb (Cabrales et al. 2010). Thus the ability of T-state PolybHbs to adequately transport oxygen in vivo was confirmed in this study (Cabrales et al. 2010).

A thorough investigation into the gaseous ligand binding/release kinetics and autoxidation kinetics of high MW T- and R-state PolybHbs was then conducted to better understand their in vivo oxidation potential and pharmacokinetics (Buehler et al. 2010). Stop flow kinetic measurements were used to determine the $O_2$ dissociation, CO association, and NO dioxygenation rate constants of 40:1 R-state and 50:1 T-state PolybHbs, all PolybHbs had MWs $>$ 500 kDa (Buehler et al. 2010). The $O_2$ dissociation rate constant increased for 50:1 T-State PolybHb and decreased for 40:1 R-state PolybHb compared to bHb, indicating that $O_2$ is released faster from the 50:1 T-state PolybHb compared to 40:1 R-state PolybHb (Buehler et al. 2010). The reported value of the CO association rate constant of 50:1 T-state PolybHb was marginally lower than that of bHb, whereas this rate constant increased for 40:1 R-state PolybHb (Buehler et al. 2010). The NO dioxygenation rate constants of both the 50:1 T-state and 40:1 R-state PolybHbs were shown to be similar to that of bHb (Buehler et al. 2010). This indicates that both PolybHbs and cell-free bHb interact similarly with NO (Buehler et al. 2010). Counter intuitively, an earlier study observed that T-state PolybHbs do not produce vasoconstriction and hypertension, which have been linked to NO scavenging (Cabrales et al. 2009). Thus since PolybHbs are capable of interacting with NO, it is hypothesized that their large size (compared to the size of the endothelial cell–cell junctions lining the blood vessel wall) prevents them from extravasating through the endothelium and scavenging NO (Cabrales et al. 2009; Buehler et al. 2010). In vivo, 50:1 T-state PolybHb underwent autoxidation at a significantly lower rate and remained in circulation longer than 40:1 R-state PolybHb (Buehler et al. 2010). This result is important, since the reduced rate of heme oxidation prolongs T-state PolybHb

oxygen delivery in vivo (Buehler et al. 2010). Therefore, high MW T-state PolybHbs seem to be an ideal HBOC due to their lack of vasoconstriction and hypertension, increased circulatory half-life, and reduced in vivo oxidation compared to R-state PolybHbs (Buehler et al. 2010).

An evaluation of T- and R-state glutaraldehyde polymerized HbA (PolyhHb) demonstrated that PolybHbs and PolyhHbs share many of the same biophysical characteristics (Buehler et al. 2010; Zhang et al. 2011). In this study, 50:1 and 40:1 T-state PolyhHbs and 30:1 and 20:1 R-state PolyhHbs were synthesized with MWs $>$ 500 kDa (Zhang et al. 2011). Both the $P_{50}$ and oxygen dissociation rate constants of T-state PolyhHbs were higher than those of hHb and R-state PolyhHbs (Zhang et al. 2011). Thus the low oxygen affinity of T-state PolybHbs and high oxygen affinity of R-state PolybHbs are maintained in T- and R-state PolyhHbs, respectively (Buehler et al. 2010; Zhang et al. 2011). Polymerization decreased the CO association binding rate constant of T-state PolyhHbs, while this rate constant increased for R-state PolyhHbs (Zhang et al. 2011). This is similar to the trends observed in T- and R-state PolybHbs (Buehler et al. 2010; Zhang et al. 2011). T- and R-state PolyhHbs also maintained their ability to interact with NO similar to HbA, regardless of Hb quaternary state (Buehler et al. 2010; Zhang et al. 2011). In addition to these kinetic experiments, the ability of PolyhHbs to deliver $O_2$ was simulated using a mathematical model of a hepatic hollow fiber bioreactor (Zhang et al. 2011). Modeling results indicated that T-state PolyhHbs were more effective at delivering oxygen to hepatocytes housed in the bioreactor compared to R-state PolyhHbs (Zhang et al. 2011). This study showed that PolyhHbs share many of the same biophysical characteristics as PolybHbs, and indicated that T-state PolyhHbs possess the best potential to serve as oxygen transporting RBC substitutes (Buehler et al. 2010; Zhang et al. 2011).

Another study evaluated the ability of PolybHbs to deliver oxygen using the same mathematical model of a hepatic hollow fiber bioreactor used to evaluate PolyhHbs (Zhou et al. 2011; Zhang et al. 2011). In this study, T- and R-state PolybHbs were synthesized at the following glutaraldehyde to bHb molar ratios: 10:1, 20:1, and 30:1, and previous results for 40:1 R-state and 50:1 T-state PolybHbs were incorporated in the hepatic hollow fiber model (Buehler et al. 2010; Zhou et al. 2011). The results of these simulations showed that all T-state PolybHbs were able to more efficiently deliver $O_2$ to hepatocytes housed in the bioreactor compared to R-state PolybHbs, further solidifying the superior oxygenation potential of T-state PolybHbs as RBC substitutes (Zhou et al. 2011).

In order to evaluate the effect of PolybHb size on oxidative tissue toxicity, T-state PolybHbs were synthesized at the following glutaraldehyde to bHb molar ratios: 10:1, 20:1, 30:1 and 40:1 (Baek et al. 2012). Guinea pigs were then subjected to a 50 % blood for PolybHb solution exchange transfusion and the pharmacokinetics of PolybHb was evaluated along with iron deposition in the spleen, liver and kidneys (Baek et al. 2012). The results of this study showed that the 30:1 PolybHb elicited less in vivo oxidation in the blood compared to the 10:1, 20:1, and 40:1 PolybHbs, that was not a function of PolybHb size (Baek et al. 2012). However, iron deposition in the kidneys decreased as a function of increasing

PolybHb size (Baek et al. 2012). Similarly, the extent of systemic hypertension decreased with increasing PolybHb size, while the circulatory half-life of PolybHb increased as a function of increasing PolybHb size until it reached a maximum for the 30:1 PolybHb (Baek et al. 2012). In light of these results, the 30:1 T-state PolybHb exhibited the best pharmacokinetics with the least iron deposition in the kidneys along with the absence of systemic hypertension upon transfusion (Baek et al. 2012). Additional studies will need to be performed to further assess the clinical safety of this material.

## 37.4 Conclusions

In summary, Palmer's group has systematically investigated the biophysical properties, and in vivo responses upon transfusion of variable sized T- and R-state PolyHbs (Cabrales et al. 2009, 2010; Baek et al. 2012; Palmer et al. 2009a; Buehler et al. 2010; Zhou et al. 2011; Zhang et al. 2011). The results of these studies have identified high MW T-state PolyHbs as a low oxygen affinity HBOC, which does not elicit vasoconstriction, hypertension, or oxidative tissue toxicity (Cabrales et al. 2009, 2010; Baek et al. 2012). In addition, high MW T-state PolyHbs are able to deliver $O_2$ in both in vitro and in vivo scenarios (Cabrales et al. 2010; Buehler et al. 2010; Zhou et al. 2011; Zhang et al. 2011). Therefore, these results set the stage for exploring the clinical potential of high MW T-state PolyHbs as RBC substitutes in transfusion medicine.

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# Chapter 38
# Acellular Hemoglobin-Based Oxygen Carrier Induced Vasoactivity: A Brief Review of Potential Pharmacologic Remedies

**Hae Won Kim, Chi-Ming Hai and A. Gerson Greenburg**

## 38.1 Introduction

Artificial oxygen carriers based on chemically modified or recombinant human or animal hemoglobins (Hb) are promising as red blood cell substitutes because they have some specific advantages over allogeneic donor red cells (Kim and Greenburg 2004). For example, they are virtually pathogen-free, can be stored at room temperature for extended periods and can be administered to recipients regardless of blood type. A couple of acellular hemoglobin-based oxygen carriers (HBOCs) are in the final stages of clinical development (Kim and Greenburg 2004; Kocian and Spahn 2008). However, regulatory approval has been hampered because some serious adverse effects including severe hypertension, cardiac and other cardiovascular events have been observed in recent clinical studies (Sloan et al. 1999; Saxena et al. 1999; Greenburg et al. 2004; Serruys et al. 2008; Moore et al. 2009; Jahr et al. 2008; Olofsson et al. 2006). Results of these clinical trials are controversial since HBOC treatments did not significantly improve mortality rate or other clinical outcome indicators over donor red cells or standard resuscitation fluids despite some clinical benefits including improved hemodynamic status and reduction in allogeneic red cell use (Hill et al. 2002; Kasper et al. 1996; LaMuraglia MD et al. 2000; Sprung et al. 2002; Carmichael et al. 2000). Debates are ongoing as to whether the lackluster results are due to poor study design, high protocol violation rates, or an inherent toxicity of HBOC itself. Because of HBOC's propensity

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to cause vasoconstriction and a higher incidence of adverse events (AEs) in HBOC treated patients than control fluid treated patients, there have been concerns that vasoconstrictive property of these products may be involved in the genesis of those AEs (Silverman et al. 2008; Natanson et al. 2008). Hypotheses and theories are abundant but a direct link showing HBOCs were the causative agents for the observed AEs has not been definitively established. It is clear, however, that most current acellular HBOC products do cause systemic and pulmonary BP elevations ('hypertensive' responses) in animals and human subjects. Both human and animal Hbs have a high reactivity with endothelium derived NO, a potent vasodilator constitutively produced by the vascular endothelium to regulate vascular tone (Martin et al. 1985, 1986). Therefore, it is plausible that Hb scavenging of endothelial NO may contribute to the observed hypertensive/vasoactivity effects. Yet, the exact mechanism of HBOC-mediated BP elevation (hypertension) has not been fully elucidated. Other plausible mechanisms have also been proposed and it is possible that multiple mechanisms may be involved depending on clinical circumstances. A recent NIH workshop recommended more fundamental studies to delineate mechanisms of HBOC-mediated vasoactivity/BP elevation and other adverse effects (Estep et al. 2008). One encouraging finding from recent preclinical and clinical studies is that the HBOC-mediated vasoactivity/BP elevation can be modulated with the use of conventional vasodilators or anti-hypertensive agents regardless of the mechanism involved. However, little information is available regarding detailed descriptions of how these agents were used (e.g., patients' conditions, doses used, outcome or complications) nor mention of mechanisms of therapeutic action. To our knowledge, there are no published reports that have systematically reviewed and discussed use of these agents for treatment of the HBOC-mediated BP elevation. Here, we review preclinical and clinical studies that used conventional or newer anti-hypertensive agents to attenuate HBOC-mediated vasoconstriction/BP elevation and discuss potential issues.

## 38.2 Nature of Acellular HBOC-mediated Vasoconstriction and BP Elevation

A recent FDA report states that "all current HBOC products or previously in development are vasoactive at the doses proposed for resuscitation or for blood replacement" (Silverman et al. 2008). Intravenous administration of most current acellular HBOC products to animals and humans have shown to increase systemic blood pressure without concomitant increase in the cardiac output, an indication that HBOC induced BP elevation is mediated by systemic vasoconstriction. However, the hypertensive responses do seem to vary with HBOC product characteristics ([Hb], MW, P50, viscosity, etc.), dose/rate, and study protocol/models used (Estep et al. 2008). Generally, BP starts to increase almost immediately, peaks within few minutes, and lasts 1–3 h after completion of HBOC infusion

depending on the dose given. The HBOC-mediated BP elevations were described as 'generally mild to moderate' and 'transient' in recent clinical trials (Jahr et al. 2008; Freilich et al. 2009).

In animal studies, HBOC-mediated transient hypertensive effects also varied with physico-chemical characteristics of different HBOCs, HBOC dose, species and protocols (Estep et al. 2008). Topload (hypervolemic) infusion or exchange (normovolemic) transfusion protocols produced generally more pronounced BP elevations than hemorrhagic shock-resuscitation protocols. In-vitro isometric contraction studies with isolated vascular segments revealed that vascular responses to a HBOC also varied among animal species and vessel types. For example, pig vessels were more sensitive than those of rats, rabbits and dogs (Freas et al. 1995; Muldoon et al. 1996; Hart et al. 1997). In addition, different blood vessel types even within the same animal species elicit substantially different contractile responses to the same HBOC treatment (Freas et al. 1995). Further, for HBOC to elicit contraction, isolated thoracic aortas from rats and rabbits require precontraction with an agonist while porcine pulmonary vessels do not (Freas et al. 1995; Muldoon et al. 1996). At comparable HBOC doses, pulmonary vessels are generally more sensitive than other vessels. Removal of the endothelium or pretreatment with $N^g$-nitro-L-arginine methyl ester (L-NAME, a nitric oxide synthase inhibitor) and certain adrenergic antagonists prevented HBOC-induced contractions (Muldoon et al. 1996; Kim and Greenburg 1997; Kim et al. 2001).

In clinical studies, HBOC-mediated hypertensive effects were also observed in patients as well as healthy subjects (Saxena et al. 1999; Jahr et al. 2008; Olofsson et al. 2006; Carmichael et al. 2000). In a multicenter clinical trial of 688 patients with surgical anemia during orthopedic surgery (Jahr et al. 2008; Freilich et al. 2009), there was significantly higher incidences of adverse events associated with BP increase in patients treated with HBOC-201 (Hemopure®, Biopure Corp., Cambridge, MA) compared with those treated with packed red cells: 17 % (60/350) vs. 7 % (22/338), respectively. In this study, the first 500 mL (65 g Hb) infusion of HBOC-201 resulted in the largest increases in the systolic and diastolic blood pressures. After the first infusion, the mean peak SBP of HBOC-201 treated patients were 143 mm Hg compared with 126 mm Hg for the packed red cell (PRBC) treated group. The mean peak systolic blood pressures (SBP) in subsequent infusions were 160 mm Hg and 151 mm Hg for HBOC-201 and PRBC groups, respectively. While 26 % of HBOC-201 treated patients experienced peak SBP of >161 mm Hg, only one patient experienced a severe BP elevation considered a severe adverse event (SAE). In comparison, only 6 % of packed red cell treated patients experienced peak SBP of >161 mm Hg. All increases in BP resolved spontaneously or with treatment (Freilich et al. 2009).

A phase II safety study with 250 or 500 ml MP4 (Hemospan®, Sangart Corp. San Diego, CA) in elderly patients undergoing orthopedic surgery produced similar results (Olofsson et al. 2006); 10.2 % (6/59) of patients who received human PEG-Hb developed hypertensive adverse events while only 3.2 % (1/31) of control solution (Ringer's acetate) patients did. In stroke patients, 25–100 mg/Kg DCLHb (HemeAssist®, Baxter Corp., Deerfield, IL) produced a rapid rise in mean

arterial pressure (MAP), which reached a maximum within 2 h after the first infusion (Saxena et al. 1999). The BP increased from 113 mm Hg at baseline to 134 mm Hg in DCLHb treated patients compared with 109 mm Hg in controls. The magnitude of the BP increases was similar for all doses but the duration of the pressor effect was dose dependent. The hypertensive reaction did not accompany clinical and radiological signs of cerebral pathologic changes. Three of the 40 (7.5 %) patients treated with DCLHb developed severe hypertension requiring pharmacologic intervention while 3/45 (6.7 %) control patients (Saxena et al. 1999). Unfortunately, the report provided neither criteria of the 'severe hypertension' nor description of pharmacologic intervention used (Saxena et al. 1999). In a Phase I study of 42 healthy adult male volunteers (Carmichael et al. 2000), 33 received 0.025–0.6 g Hb/Kg o-raffinose Hb (Hemolink®, Hemosol, Inc., Toronto, Canada); dose-dependent MAP increases were observed with a plateau occurring 14 % above the baseline at 0.1 g Hb/Kg. In patients undergoing coronary artery bypass graft (CABG) surgery, hypertension (defined as SBP >140 mm Hg) occurred in 16/28 (57.2 %) o-raffinose Hb (25–75 g Hb) treated patients versus in 9/32 (28.1 %) control patients (Hill et al. 2002).

It appears that HBOC elicits a more pronounced hypertensive effects in normovolemic patients than in hypovolemic patients. In a Phase II study with patients undergoing an elective percutaneous coronary intervention procedure, 31 % (9/29) of HBOC (15 or 30 g Hb) treated patients developed a severe hypertension (SBP >180 mm Hg) while none of the artificial colloid treated patients (0/16) had hypertension reported as adverse events (AEs) (Serruys et al. 2008). The severe BP elevations were treated with intravenous nitroglycerine or other unspecified antihypertensive drugs as necessary.

## 38.3 Is There an Acceptable Level of Vasoactivity?

In most patients with no significant underlying cardiovascular pathology, HBOC-mediated BP elevations are reported to be moderate and transient and did not require therapeutic intervention while some patients who developed severe BP elevations did require pharmacologic interventions (Saxena et al. 1999; Jahr et al. 2008; Kasper et al. 1996; LaMuraglia et al. 2000; Sprung et al. 2002; Freilich et al. 2009). For patients with severe hemorrhagic shock or ischemic stroke, the potential beneficial effect of HBOC-mediated moderate BP elevation is being debated (Sloan et al. 1999; Mistri et al. 2006). But individual patient's clinical condition and underlying pathologies should be carefully considered as patients with significant cardiovascular pathologies are at higher risk for hypertension induced serious adverse events (e.g., cardiac events, stroke, hemorrhage, organ damage, etc.). Therefore, if a patient is hypertensive, diabetic or elderly with significant underlying cardiovascular pathology, post-HBOC infusion BP should be carefully monitored and a proper anti-hypertensive therapy be instituted promptly if deemed necessary. Whether or when to treat HBOC-mediated BP

elevation would depend largely on the degree of BP elevation, patient's condition, the underlying pathophysiology, and the indication for HBOC. Of note, when administering antihypertensive agents to anesthetized patients, caution should be exercised as halothane and certain local anesthetics have been reported to mask HBOC mediated BP responses (Bone et al. 1999).

## 38.4 Key Mechanisms Proposed for the HBOC-mediated Vasoconstriction/BP Elevation

There are several proposed mechanisms for the HBOC-mediated vasoconstriction/BP elevation; the leading hypotheses are briefly discussed below.

### *38.4.1 Vasoconstriction Via Hb Scavenging of Endothelial NO*

Under normal conditions, NO is constitutively produced in the vascular endothelium by the action of endothelial nitric oxide synthase (eNOS or NOS-3). Once diffused to the smooth muscles, NO activates soluble guanylyl cyclase (sGC) to produce elevated level of cGMP which results in smooth muscle relaxation. One popular hypothesis for the HBOC-mediated vasoconstriction and BP elevation is HBOC inactivation of endothelium derived NO, a potent vasodilator that mediates GC-cGMP dependent vascular smooth muscle relaxation. Because Hb has an intrinsically high reactivity with NO, the presence of large amounts of acellular Hb/HBOC in the vascular lumen could interrupt endothelium-derived NO flux into the smooth muscle resulting in vascular contraction. In addition, because of smaller particular size (<5–10 nm), some HBOCs could extravasate through the endothelial fenestrations into the subendothelial space allowing closer contact with endothelial NO. Some HBOCs are too large in particle size to pass through the fenestrations. However, acellular HBOC dissolved in the plasma is several hundred times more reactive with NO than native Hb compartmentalized in the protective red blood cells (Gladwin et al. 2004). In addition, only Hbs and HBOCs with ferrous hemes elicit contractions in the vessels with intact functional endothelium supporting the hypothesis (Kim and Greenburg 1997, 2000; Pawson et al. 2007).

### *38.4.2 Oxygen Dependent Autoregulatory Vasoconstriction*

It has been reported that two Hb preparations (native cell-free Hb and PEG-Hb) that have similar NO binding rates elicit notably different hemodynamic effects (Rohlfs et al. 1998). In this study, Hb solutions that exhibited transient or no

significant increase in BP had higher NO binding affinities than Hb solutions that sustained BP increases. Based on these observations, they claimed that NO scavenging at the heme site cannot be the cause of BP increases but rather must be due to other physiologic mechanisms. They hypothesized that acellular low $O_2$ affinity ($P_{50} \sim$ 50) HBOCs may lead to excessive oxygen offloading in the arterioles upstream from the capillary beds causing reactive arteriolar vasoconstriction and decreased functional capillary density ($O_2$ dependent autoregulatory vasoconstriction). (Rohlfs et al. 1998; Vandegriff and Winslow 2009; Vandegriff et al. 2003).

### *38.4.3 Hb Stimulation of Endothelin-1 Release*

In preclinical and clinical studies, intravenous administration of DCLHb caused dose-dependent increases in plasma levels of endothelin-1 (ET-1), a potent vasoconstrictor (Gulati et al. 1995, 1996, 1997; Saxena et al. 1998). The pressor response and other cardiovascular effects of DCLHb could be attenuated by pretreatment with BQ-123 and FR-139317, ETA-receptor antagonists (Gulati et al. 1996) suggesting that the HBOC-induced vasoconstriction/BP elevation may involve stimulation of ET-1 release. Interestingly, infusion of an ultra-large molecular weight based HBOC (20 MDa) in animals with focal cerebral ischemia caused no systemic hypertension but cerebro-arteriolar vasoconstriction that was reported to be mediated primarily by ET-1 (Cao et al. 2009). Further, ET receptor antagonists attenuated pressor effects of DCLHb in rats (Rioux et al. 1999). These results suggest that ET-1 may play a more significant role in certain vascular beds and animal models (Gulati et al. 1997; Rioux et al. 1999).

In addition, other mechanisms have also been proposed for the HBOC-mediated vasoconstriction and BP elevation including Hb stimulation of adrenergic vasoconstrictive mechanisms (Gulati and Rebello 1994), and pseudo-ACE activity of Hb (Simoni et al. 2007). Although one key mechanism may function as a dominant player, other mechanisms may also contribute depending on clinical conditions and underlying pathologies of the patients involved.

## 38.5 Potential Pharmacologic Remedies for HBOC-mediated Vasoconstriction and BP Elevation

In recent clinical trials, hypertensive responses following HBOC administration were described as ‘mild and transient’ and did not require any therapeutic intervention (Jahr et al. 2008; Freilich et al. 2009). However, some patients did develop clinically serious post-HBOC administration hypertension serious enough to be reported as AEs/SAEs but no information is available how many of these patients actually needed treatment and, if treated, specifics of therapy given. These patients

and those with serious underlying cardiovascular pathology and limited reserve capacity should be treated promptly with proper anti-hypertensive therapies. The conventional anti-hypertensive therapies for hypertensive emergencies/urgencies as defined by the Joint National Committee (Chobanian et al. 2003) should generally be applicable to the treatment of HBOC-mediated acute BP elevations as well. In fact, HBOC-mediated critical BP elevations do seem to occur more frequently in hypertensive patients with underlying conditions that tend to elicit BP elevation (e.g., diabetes, renal disease) (Serruys et al. 2008; Jahr et al. 2008). Conventional anti-hypertensive guidelines and therapies including diuretics, rennin-angiotensin system antagonists, $\alpha$- and/or $\beta$-blockers and $Ca^{++}$ channel blockers are viable treatment options (Chobanian et al. 2003). As described previously, in clinical trials HBOCs typically caused an almost immediate BP rise after start of intravenous infusion. The BP rise did not generally exceed 25 mm Hg above the pretreatment values even at relatively high topload (for non-hypovolemic indications) doses (e.g., 30 g Hb) (Serruys et al. 2008). However, in relatively small number of patients, the post-HBOC BP elevations were severe (some may qualify as 'hypertensive urgencies or emergencies') enough to be reported as hypertensive SAEs (Serruys et al. 2008; Jahr et al. 2008; Silverman et al. 2008; Freilich et al. 2009). Management of HBOC-induced severe BP elevation (hypertensive emergencies or urgencies) may require faster acting intravenous agents to quickly lower the blood pressure to a safer level (Varon and Marik 2000). Some established anti-hypertensive therapies as well as new approaches that seem relevant to the management of HBOC-mediated BP elevation are discussed below.

Today, there are many highly effective drugs available for treatment of a chronic hypertension (Chobanian et al. 2003). Only a couple of these agents have actually been used in HBOC clinical trials. Furthermore, virtually no study have been done systematically to study safety and effectiveness of these drugs when used for prevention or treatment of HBOC-mediated acute BP elevations even in preclinical studies. In theory, any agent that increases NO availability to the vascular smooth muscle or causes vascular relaxation through other mechanisms would be effective in modulating HBOC-mediated vasoconstriction/BP elevation. However, selection of an appropriate therapeutic agent must be based on careful consideration of the individual patient's clinical condition, underlying pathophysiology, and potential interaction with HBOC directly or indirectly. Of note, in patients with severe hypertension, the recommended treatment objective for patients with 'hypertensive emergencies' is not immediate normalization of BP but rather to reduce BP to a more safely manageable level to prevent or minimize end-organ damage followed by gradual return to a normal level (Varon and Marik 2000). In patients with 'hypertensive urgencies', it is recommended that BP be lowered gradually over a period of 24–48 h, usually with oral medications. Rapid uncontrolled BP reduction may result in cerebral, myocardial and renal ischemia/infarct (Prisant et al. 1993; Ziegler 1992). Here we discuss advantages and disadvantages of some selected anti-hypertensive agents, potentially useful in the management of acute severe elevations in BP that may occur following HBOC administration.

### *38.5.1 Adrenergic Agonists/Antagonists*

In studies with isolated arterial vessel segments and whole animals in vivo, Hb/HBOC in nM ~ μM concentration range were shown to elicit vascular contraction (Freas et al. 1995; Muldoon et al. 1996; Hart et al. 1997; Kim and Greenburg 1997, 2000). In these experiments, pretreatment with phentolamine or prazosine (α-adrenergic antagonists) prevented Hb/DCLHb-mediated contractions or pressor effect (Kim et al. 2001; Gulati and Rebello 1994). However, phentolamine pretreatment did not prevent Hb-mediated contraction in vessel rings precontracted with KCl suggesting adrenergic activation does not appear to be a pre-requisite. In cervical sectioned and bilateral adrenectomized rats, intravenous DCLHb still caused significant systemic BP elevations (Gulati and Rebello 1994). In these animals, however, pretreatment with phenoxybenzamine and prazosin (α-1 adrenergic antagonists) blocked DCLHb mediated pressor effect. In addition, the DCLHb-mediated BP increases were also blocked by yohimbine (alpha-2 adrenergic antagonist) pretreatment (Sharma and Gulati 1995) suggesting that DCLHb-mediated pressor effect may be partially mediated through alpha-2 adrenergic mechanism. Alternatively, a centrally acting α-adrenergic agonist (e.g., clonidine) may also be useful as it stimulates presynaptic alpha-2 receptors that results in inhibition of norepinephrine release and lower vascular tone. Clonidine is currently used for treatment of hypertensive urgencies (Varon and Marik 2000). Because oral clonidine lowers BP more gradually, it may be suitable for those patients that a rapid BP reduction is not necessary. Therefore, α-adrenergic antagonists/agonists may be potentially useful in attenuating HBOC-mediated vasoconstriction and BP elevation. However, their use may be complicated in certain patients because adrenergic vasopressors are often used in severely hypotensive hemorrhagic patients (Herget-Rosenthal et al. 2008).

### *38.5.2 Nitrovasodilators*

#### 38.5.2.1 Organic Nitrates

One strategy to alleviate HBOC-mediated vasoconstriction, is to supplement NO towards the normal level with an exogenous NO source. Nitroglycerine or glyceryl tri-nitrate (GTN), the most commonly used of all organic nitrates, is a potent vasodilator widely used clinically for coronary vasospasm and other conditions. Its vasodilatory mechanism has not been fully elucidated but, recently, it was reported to elicit vasodilation via NO produced by action of mitochondrial aldehyde dehydrogenase (Chen et al. 2002; Chen and Stamler 2006) which catalyzes reduction of GTN to generate nitrite and 1,2-glycerate dinitrate. The nitrite so generated is proposed to be further metabolized to generate NO which then elicit GC-cGMP mediated vasodilation. However, this theory has recently been

challenged as GTN mediated relaxation was not observed with concomitant NO fluorescence nor inhibition of $O_2$ consumption by vascular mitochondria (Nunez et al. 2005). Nevertheless, GTN may be an effective therapeutic agent for modulation of the HBOC-mediated vasoconstriction and BP elevation. In rat aortic ring preparations, GTN significantly reduced acellular Hb-mediated contraction (Kim and Greenburg 2000). In a recent clinical study in patients undergoing an elective percutaneous coronary intervention procedure, critical elevations of BP (defined as SBP >180 mm Hg) occurred in 31 % of patients following treatment with HBOC-201 (Serruys et al. 2008). In these patients, BP elevations were generally managed with intravenous GTN. As GTN tolerance often occurs, one patient was not responsive to GTN treatment and required nifedipine (calcium channel blocker) to control the BP.

Of note, nitrates are known to oxidize Hb creating ferric Hb or metHb and instances of intraerythrocytic methemoglobinemia following administration of GTN and other organic nitrates have been documented (Coleman and Coleman 1996). Because of the presence of large amounts of metHb-reductase (NADH-cytochrome b complex) and other reducing agents in the red blood cells of normal people, toxic levels of metHb accumulation is relatively rare. However, intravenously administered HBOCs circulate in the plasma phase where metHb reducing capacity is relatively low. Therefore, plasma metHb levels could accumulate more easily in patients with plasma HBOC following administration of GTN or other nitro-drugs with a high oxidation potential. In a clinical trial of surgical patients administered up to 2.0–2.5 g Hb/Kg of HBOC-201 (total of approximately 180 g Hb), metHb level was < 2 % at post-operative day 1 but reached a peak of approximately 7 % (Sprung et al. 2002). In a more recent clinical study of orthopedic surgery patients, cumulative HBOC-201 doses of up to 330 g (11 units) were given over a 6-day period (average of 136 g Hb). The mean metHb levels ranged 0.6–5.8 % after each infusion including two patients whose peak metHb levels reached 11 and 14 % but none of the patients exhibited symptoms or required treatment (Jahr et al. 2008). In addition, when significant amount of metHb or ferric HBOC is formed, the total oxygen binding/carrying capacity of HBOCs is also reduced as metHb is unable to bind and carry $O_2$. Of note, until metHb level reaches >30 %, clinical symptoms may not appear. When metHb level reaches over 50 % (fatal if >70 %), cardiovascular dysfunction and neurologic deficits may occur as the blood is unable to transport sufficient $O_2$ to meet metabolic demand. In such case, metHb level can generally be reduced by administering intravenous methylene blue (Clifton and Leikin 2003).

In addition, although GTN and other organic nitrates are useful in controlling severe hypertension in patients with cardiac ischemia, they may not be the best choice in other patients (Bussmann et al. 1992). GTN causes arterial as well as venodilation thus reducing preload and cardiac output. Besides, many patients develop tolerance to GTN making it less effective with repeated doses. For these drugs, optimal dosing in hemodynamically unstable patients poses significant challenges.

#### 38.5.2.2 Sodium Nitroprusside

Sodium nitroprusside (SNP) is a potent vasodilator often used for treatment of severe acute hypertension. However, in rats treated with clinically relevant doses (>1 g/Kg) of DCLHb, potency of SNP potency was significantly reduced (Erhart et al. 2000) implying higher doses of SNP would be required to control severe hypertension in the presence of high levels of plasma HBOC. SNP is a potent arterial and venous vasodilator and intravenous administration is the clinical treatment of acute hypertension or for hypertensive crisis (defined as SBP >160 mm Hg or DBP >10 mm Hg) (Varon and Marik 2000). Its pharmacologic action is thought to be due to spontaneous degradation to release NO in the blood. But recent reports suggest that it, too, may go through biotransformation in the vascular smooth muscle and endothelial cells to produce NO (Kowaluk et al. 1992; Aldini et al. 2006).

Of note, when treating HBOC-mediated BP elevation with SNP, caution should be exercised since SNP releases highly toxic cyanide ions ($CN^-$) causing potentially lethal cyanide poisoning. Thus, the maximal dose should generally not exceed normal hepatic cyanide clearance rate of 2 μg/kg/min (Friederich and Butterworth 1995). Small amount of cyanide in the blood is rapidly metabolized by the liver to less harmful thiocyanate by the action of the enzyme *rhodanase* (requires sulfur donor such as thiosulfate). Thiocyanate is then excreted in the urine by the kidney. However, in the absence of sufficient sulfur donor, cyanide ions could quickly reach toxic levels (>1 mg/ml). Thus, the duration of treatment should generally not exceed 72 h and plasma thiocyanate concentrations should be carefully monitored. SNP also degrades upon exposure to light to produce cyanide. In patients with renal failure or patients with the deficient in rhodanase enzyme or low sulfur donor levels, SNP should not be used as it could cause fatal cyanide poisoning.

### *38.5.3 $Ca^{++}$-Channel Blockers*

Calcium channel blockers are commonly used anti-hypertensive drugs which generally reduce cardiac and vascular smooth muscle cytosolic $Ca^{++}$ leading to decreased cardiac output and vasodilation. The $Ca^{++}$-channel blockers are further classified into dihydropyridines (e.g., nicardipine, nifedipine), phenylalkylamines (e.g., verapamil) and benzothiazepines (e.g., diltiazem). With exception of few, most of $Ca^{++}$-channel blockers are typically formulated to be administered orally due to low water solubility. For management of acute severe HBOC-mediated BP elevation, fast acting intravenous or sublinguial formulations would be more desirable. In fact, a recent Phase II study of HBOC-201 reported that intravenous administration of nifedipine successfully controlled critical hypertension (>180 mm Hg) which did not respond to an intravenous nitroglycerine (Serruys et al. 2008).

In a study with awake spontaneous hypertensive rats, 10 % topload infusion of o-raffinose Hb resulted in significant increase in MAP (36 mm Hg) at 10 min after the start of infusion. In these animals, pretreatment with 10 mg/kg nifedipine by gavage reduced subsequent MAP rise by 50 % (Ning et al. 2000). Similarly, in anesthetized rats, DCLHb induced pressor effect was significantly inhibited by nimodipine and verapamil (Rioux et al. 1998). However, the bradycardiac effect of DCLHb was not affected by nimodipine. These results suggest that treatment with $Ca^{++}$-channel blockers may prevent/modulate HBOC-mediated hemodynamic effects.

### *38.5.4 Angiotension Converting Enzyme Inhibitors and Angiotension-II Receptor Blockers*

The renin-angiotension system is a powerful endocrine blood pressure regulating mechanism. When BP is low, kidneys produce renin that activates angiotension-I production from inactive angiotensinogen. By action of an angiotension converting enzyme (ACE), angiotension-I is then converted to angiotension-II, a potent vasoconstrictor. ACE inhibitors (ACEIs) block production of angiotension-II thereby effectively reduce BP. Currently, ACEIs are one of the major categories of drugs used for treatment of hypertension (Matchar et al. 2008). Interestingly, a recent study reports that cell-free Hb exerts an ACE-like activity when activated by plasma hydrogen peroxide (Simoni et al. 2007). The assertion was based on the observation that angiotension-I was converted to angiotension-II in the presence of cell-free Hb. If confirmed, this mechanism may also contribute to the Hb-mediated vasoconstrictive effect. Therefore, drugs that prevent or antagonize the action of rennin-angiotensin system including ACEIs and angiotensin-II receptor blockers (ARBs) may potentially be useful in modulating the HBOC-mediated BP elevation. Use of ACEIs is generally recommended in patients with compromised renal function but some new data seems to indicate that ACEI may elicit end-stage renal failure (Khan et al. 2009; Woo et al. 2006; Suissa et al. 2006).

### *38.5.5 Phosphodiesterase Inhibitors*

Phosphodiesterases (PDEs) breakdown second messenger cyclic nucleotides, cGMP and cAMP which are ubiquitous in virtually all cells and are involved in many different functions including vascular smooth muscle relaxation. Therefore, non-specific PDE inhibitors (PDEIs) have not generally been used in the treatment of hypertension per se. Nevertheless, some PDEIs including a recently discovered highly selective PDEI (e.g., sildenafil) have shown to reduce HBOC-mediated vasoconstriction/hypertensive effects.

#### 38.5.5.1 Sildenafil

Sildenafil is a PDE-5 selective inhibitor (PDE5I) that prevents degradation of cGMP in the corpus cavernosum and is currently used primarily as remedies for erectile dysfunction. But they also lower systemic and pulmonary hypertension making them potentially useful in modulating HBOC-mediated hypertensive responses. In fact, results of a recent animal study seem to indicate that HBOC-mediated negative vascular responses can largely be overcome by concomitant PDE5I treatment (Gotshall et al. 2009).

#### 38.5.5.2 Papaverine

Papaverine is an opium alkaloid used for treatment of gastrointestinal, cerebral and coronary vasospasms. The vascular relaxation mechanism of papaverine action has not been fully elucidated but is generally considered to act via nonspecific inhibition of cAMP-phodiesterase. In an in vitro experiment, papaverine significantly attenuated or reversed Hb-induced contractions of isolated rat thoracic aortic ring preparations (Kim and Greenburg 1997). These results suggest that some selective PDE inhibitors may have potential in attenuating or reversing HBOC-mediated vasoconstriction/BP elevation but further studies are needed to evaluate their safety and efficacy.

### *38.5.6 Sodium Nitrite*

Relatively high plasma nitrite levels have been reported in the plasma of healthy individuals (values for brachial artery and antecubital vein: 322–540 nM and 305–466 nM, respectively) (Gladwin et al. 2000; Lauer et al. 2001). Therefore, nitrites are considered as a possible endogenous NO reservoir for blood flow regulation and other functions (Gladwin et al. 2006). Mechanisms for the in vivo conversion of nitrite to NO have been proposed. Recently, deoxyhemoglobin is proposed to serve as a nitrite reductase under hypoxic conditions (Cosby et al. 2003). To test the hypothesis, 18 normal subjects were infused with up to 1 mmoles of sodium nitrite over about a 30 min period into the forearm brachial arteries which resulted in a systemic concentration of 16 μM. Infusion of nitrite caused an immediate increase in forearm blood flow by 175 % and concomitant reduction in the systemic BP of ~7 mm Hg (Cosby et al. 2003). This reduction in BP occurred with rapid formation of iron-nitrosyl Hb and, to a lesser extent, S-nitroso Hb in the blood. MetHb levels increased significantly from the baseline but still remained relatively low (from ~0.2 to ~0.5 %). In a more recent study, 20 normal human volunteers were given intravenous sodium nitrite to determine

endocrine effect, pharmacokinetics, and tolerance (Dejam et al. 2007). In this study, the highest dose given was 110 μg/kg/min which resulted in whole blood nitrite concentration of ~850–900 μM. Forearm blood flow increased in a dose dependent manner from 2.8 to 12.3 ml/min/100 ml tissue and plateaued at around 300 μM nitrite. Plasma nitrite concentration increased from 0.13 to 26.1 μM with mean clearance of 0.95 L/min ($T_{1/2}$ = 42 min). Plasma nitrate concentration increased from 18 to 64 μM with T1/2 of 6 h. Systemic nitrosyl Hb levels increased from 0.14 to 7.9 μM with apparent T1/2 of 53 min. These changes were coincided with decrease in MAP from 97 to 86 mm Hg with a slight but non-significant increase in the heart rate (68–76 beats per minute). Of note, the MAP remained significantly lower than baseline for 2 h after the nitrite infusion was completed. Whole blood metHb levels increased from 0.7 to 3.2 % of total Hb. The highest metHb level was reached 20 min after completion of nitrite infusion with $T_{1/2}$ of 78 min.

In a recent Phase I/II clinical trials, patients with sickle cell disease (Mack et al. 2008) were given 3.2–320 μmoles of sodium nitrite administered through the brachial arterial line (1 ml/minute of 0.4–40 μM sodium nitrite over about 8 min) which resulted in 7.9–77 % increase in forearm blood flow but without significant drop in systemic BP. Dose dependent metHb increases were observed following nitrite infusion reaching peak regional venous concentration of 4 % at the highest dose tested (40 μM). Aside from nausea in one patient, none of the patients exhibited ill-effects including clinical signs of hypoxia such as cyanosis or shortness of breath. However, all of the metHb levels reported were concentrations within the erythrocyte compartment where high level of metHb reduction activities are present. Intravenously administered acellular HBOCs circulate in the plasma phase where metHb reduction activity is relatively low. Therefore, presence of high level of nitrite would facilitate conversion of ferrous HBOC to non-oxygen carrying ferric HBOC (metHBOC). In fact, for this reason, sodium nitrite is a FDA approved treatment for a cyanide poisoning as it produces metHb that neutralizes toxic cyanide ion by forming a non-toxic cyanometHb (Baud 2007). If substantial amount of HBOC is converted to metHBOC by nitrite, however, oxygen carrying capacity of HBOC will be reduced accordingly raising the possibility of insufficient oxygen supply despite vasodilation and improved blood flow. In addition, optimal dosing of nitrite would be extremely challenging especially in patients with significant underlying pathologies. In a recent animal study, a single bolus 30–100 nmol sodium nitrite (resultant blood nitrite level = 1.2 μM) at the onset of HBOC-201 resuscitation following trauma-hemorrhage prevented HBOC-201-mediated hypertension while resuscitation with nitrite plus Ringer's lactate did not alter mean arterial pressure suggesting a possible role of HBOC-201 as a nitrite reductase (Rodriguez et al. 2009). It was reported that nitrite doses of <100 nmol did not significantly increase plasma metHb level beyond that observed with HBOC-201 alone (2–3 % at 2 h post-resuscitation). Based on these findings, nitrite has been proposed as a potential adjunctive therapy to prevent HBOC-mediated hypertension (Rodriguez et al. 2009). Of note, however, 10 μmol nitrite did increase metHb level beyond that observed with HBOC-201 alone while

100 μM caused death during resuscitation. Further, in a study with healthy human volunteers, infusion of 180 μmol of sodium nitrite alone (measured systemic blood nitrite concentration, 16 μM) caused a decrease in systemic MBP of only ~7 mm Hg (Cosby et al. 2003). At this nitrite concentration without HBOC, measured serum metHb level was approximately 1.5 %. Therefore, in the presence of a HBOC, a substantially higher dose of nitrite must be used to prevent/attenuate severe HBOC-induced BP elevations (ΔBP >60 mm Hg) observed in some recent clinical trials (Serruys et al. 2008; Jahr et al. 2008; Freilich et al. 2009). Under such conditions, a much higher HBOC oxidation (metHb formation) may occur. Therefore, clinically efficacy and safety of nitrite therapy will have to be determined in human studies. In addition, the intravascular oxidation rate of acellular Hb/HBOC in humans may be higher than that observed in mice since humans are incapable of producing endogenous ascorbic acid, an important anti-oxidant that contributes to maintaining Hb in the functional ferrous state (Buehler et al. 2007). Unlike rats and mice, humans are unable to produce endogenous ascorbic acid due to evolutionary loss of hepatic L-gulonolactone oxidase.

Alternatively, sodium nitrite can also be administered via inhalation. In a recent study of newborn lambs with hypoxic pulmonary hypertension, aerosolized sodium nitrite was administered through a ventilator (30 mg nitrite in 5 ml buffered saline over 20 min) (Hunter et al. 2004). Nebulized nitrite inhalation elicited a rapid and sustained reduction in pulmonary pressure without measurable changes in the systemic blood pressure. Interestingly, it was reported that this means of nitrite delivery did not cause clinically significant increase in blood metHb levels. Immediate detection of NO in the exhaled gas and nitrosyl Hb formation in the blood supports the hypothesis that the pulmonary vasodilation was effected by NO produced possibly through nitrite reduction. If validated, this mode of nitrite delivery may be particularly useful in attenuating HBOC-mediated pulmonary hypertension.

### *38.5.7 Inhaled NO*

Inhalation of gaseous NO has been reported to be effective in the treatment of persistent pulmonary hypertension as this mode of administration preferentially delivers NO to the pulmonary circuit eliciting selective pulmonary vasodilation (Hunter et al. 2004; Romand et al. 1994; Sefton and Muldoon 1999; Bloch et al. 2007). To test whether inhalation of NO modulates the hypertensive effects of HBOC, animals were subjected to breathe low doses of NO (iNO, 5–80 ppm) before or during the HBOC administration. In anesthetized pigs, pulmonary hypertension elicited by 200 mg/kg 10 % α-α crosslinked Hb was counteracted by repeated low doses of inhaled NO (5 ppm × 10 min + 10 min rest + 10 ppm x 10 min) (Figueiredo et al. 1997). However, more recent studies indicate that longer exposure and/or higher dose of iNO may also have extra-pulmonary effect as NO is known to form S-nitroso compounds with variety of proteins under

normal and pathologic conditions (Gow 2006). In anesthetized dogs, continuous inhalation of 80 ppm NO prevented systemic vasoconstriction elicited by cell-free plasma hemoglobin (as a result of intravascular hemolysis) released by intravenous water administration (Minneci et al. 2005). However, Hb administration while continuous breathing of NO caused high rate of plasma Hb oxidation to methemoglobin which has much lower reactivity with NO. Therefore, significant metHb formation in this study might also have contributed to the absence of vasoconstriction. Of note, metHb does not bind $O_2$ as well as NO making it an ineffective oxygen carrier. Interestingly, in a recent study with awake mice and lambs, pretreatment with 80 ppm iNO for 15 min or 200 ppm for 7 min prevented acellular Hb or HBOC-201 mediated systemic hypertension without significant increase in metHb levels (Yu et al. 2008). If validated, this mode of NO administration may be useful in modulating HBOC-mediated pulmonary and systemic BP elevation in patients undergoing scheduled surgical operations and elective ischemic rescue procedures. However, in patients undergoing emergency surgeries or in severe hypovolemic shock, pretreatment with iNO before HBOC administration may not be practical. Nonetheless, inhalation of NO should be investigated further for their safety and effectiveness in modulating HBOC-mediated pulmonary and systemic BP elevation.

## 38.6 Issues in the Use of Nitrovasodilators, Nitrites, and iNO/Nebulized Nitrites with HBOC

Rodriguez et al. (2009) recently proposed that the HBOC-mediated hypertension/vasoconstriction can be modulated by co-administration of HBOC with sodium nitrite based on the theory that Hb also possesses nitrite reductase activity thereby reducing nitrite into vasodilating NO. However, there are also problems with this approach. First, because of very high affinity of NO to ferrous heme–iron ($10^7$/M/sec), the liberated NO will be captured immediately by the deoxyHb limiting its availability to smooth muscle cells where NO mediates vascular relaxation via the GC-cGMP mediated mechanism. Second, nitrite is a known Hb oxidant that could convert both erythrocytic and acellular Hbs to non-oxygen carrying ferric metHb (Coleman and Coleman 1996; Minneci et al. 2005). Acellular HBOCs in the plasma phase are particularly susceptible to oxidation lacking anti-oxidant enzymes normally present in red blood cells. If the extent of HBOC oxidation is significant, the oxygen carrying capacity of the infused HBOC will be proportionately reduced thus potentially compromising its intended efficacy.

Of note, because organic nitrovasodilators, nitrites and iNO are known oxidizers of Hb, metHb levels must carefully be monitored. In patients who are highly hemodiluted with a HBOC, a clinically significant level of plasma metHb/metHBOC may lead to insufficient oxygen delivery to tissues. If clinical signs of

hypoxia are present, intravenous administration of Hb reducing agent such as methylene blue should be considered (Clifton and Leikin 2003).

In addition to the pharmacologic agents discussed above, there are numerous other highly effective antihypertensive agents that are potentially useful in preventing/attenuating the HBOC-mediated BP elevations (e.g., $K^+$-channel openers, hydralazine, pentoxyfylline and others). However, they have never been tested in the modulation of HBOC-mediated BP elevation and need to be investigated. Some desired characteristics of an ideal anti-hypertensive agent for use in prevention or treatment of HBOC-induced BP elevation include a rapid therapeutic action, low tendency to develop tolerance and a low HBOC oxidation potential, and a wide margin of safety.

## 38.7 Conclusions

Most acellular Hb based HBOCs appear to cause vasoconstriction and BP elevation. Although relatively rare, HBOC-induced severe BP elevation (SBP >180 mm Hg) requires prompt intervention to minimize consequences especially in patients who are diabetic, hypertensive or with other serious underlying cardiovascular pathologies. The mechanism(s) for HBOC-mediated vasoconstriction and BP elevation has not been fully elucidated. Regardless of mechanisms, however, HBOC-mediated vasoconstriction and BP elevation appears to be manageable by various conventional and new pharmacologic agents including nitrovasodilators, adrenergic receptor blockers, calcium channel blockers, ACE inhibitors, PDE inhibitors and inhaled NO gas. Whether and when to treat HBOC-mediated BP elevation and the selection of appropriate anti-hypertensive therapy may be debated but must be based on individual patient's clinical condition, medical history, and underlying pathology. In addition, because titration of HBOC-mediated BP elevation could be very challenging in hemodynamically unstable patients, pharmacologic anti-hypertensive treatment should only be performed in settings where sophisticated vital sign monitoring/resuscitation resources are available. In conclusion, HBOC-mediated vasoactivity and hypertensive response may be manageable by use of selected conventional anti-hypertensive pharmacologic agents along with some new emerging vasodilatory therapeutics. However, very little information is available regarding safety and effectiveness of these approaches. For the current leading HBOC products to move forward through the regulatory approval, it would be worthwhile to explore these approaches to prevent or attenuate HBOC-induced adverse vasoactivities.

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# Part IX
# A Call for Collaborative Research and Development

# Chapter 39
# International Consortium for Development of Hemoglobin-Based Oxygen Carriers, Oxygen Therapeutics and Multifunctional Resuscitation Fluids–A White Paper

**Hae Won Kim, Jonathan S Jahr, Andrea Mozzarelli and Hiromi Sakai**

## 39.1 Background

Today, allogeneic donor blood transfusion has evolved as a life-saving treatment for many acute anemic conditions. In developed countries, safe donor blood supply is generally adequate for routine clinical demands. However, in situations where demand greatly exceeds supply (e.g., natural or man-made massive disasters), matched donor blood is not immediately available (e.g., remote locations, battlefield, a rare blood type) or blood transfusion is not an option (e.g., certain religious group or patients with an unusual antibody status), currently there is no alternative treatment.

Based on the post-WWII experience, it is estimated that approximately 20 % of battlefield casualties are potentially salvageable (IOM 1999). The single most cause of death in battlefield casualties is hemorrhage. Therefore, there is greatest opportunity for reducing morbidity and mortality in this group if a safe and battlefield usable 'blood substitute' is available. In recognition of the potential life

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saving benefit, the US Department of Defense, Office of Naval Research, is supporting development of a multifunctional resuscitation fluid (MRF) that may contain a low volume electrolyte solution, an oxygen carrier and coagulation factor(s) in a ready to use battlefield friendly package (ONR 2010).

Of note, blood transfusion carries a risk of disease transmission (e.g., AIDS, hepatitis, malaria, STD, etc.) and certain non-infectious risks (e.g., clerical errors, transfusion reactions, TRALI, immuno-modulation). In sub-Saharan Africa, supply of safe donor blood is scarce because of high prevalence of HIV/AIDS (as much as 30 % in some countries or approx 25 million people) and other transmittable diseases among the donor population (e.g. malaria, trypanosomiasis, leishmaniasis) and inadequate donor blood screening due to limited resources (WHO 2008). Moreover, more than half a million women die each year of severe post-partum hemorrhage representing up 50 % of maternal death in some countries in Africa and Asia due to shortage of safe blood (WHO 2007). Even in the developed countries, safe donor blood is in greater demand as elderly population increases (who more likely to have surgery requiring transfusion) while eligible donor pools decrease due to stagnation in population growth and emergence of newly identified transfusion transmittable pathogens (e.g., vCJD, H1N1 and West Nile viruses, etc.) (Alter and Klein 2008).

Further, allogeneic donor blood can only be used in patient with compatible antibody status requiring typing and crossmatching before use. Donor blood is limited in supply and can only be stored for 5 weeks under refrigerated conditions. In addition, there is ongoing debate that blood transfusion (especially with older blood) may be harmful in certain situations (Alter 2008; Stowell 2010).

Considering these facts, there is a great need to develop universally compatible and readily available alternatives to allogeneic donor blood (red blood cells) for use especially when transfuable blood is not available or an option.(Weiskopf and Silverman 2013). A ‘red blood cell substitute’ that is safe and effective in saving lives by adequate oxygen delivery and tissue oxygenation preserving vital organ functions during the severe hypovolemia and other acute anemic conditions would be highly desirable.

## 39.2 Development Status of HBOC, Oxygen Therapeutics (OT) and Multi-Functional Resuscitation Fluids (MRFS)

Over the last 30 years, hemoglobin-based oxygen carriers (HBOCs) have been in development as safe and clinically effective therapeutics (‘red cell substitutes’) for treatment of hemorrhagic shock, acute anemia, ischemia and other conditions. For several HBOC candidates, preclinical studies were generally positive and some leading products have been tested in Phase III clinical trials, a final stage of development process (reviews by Kim 2004; Jahr 2011). However, observations of some serious adverse events (SAEs) including severe hypertension, MI, stroke and

**Table 39.1** FDA summary of adverse events reported in HBOC clinical trials (modified from Silverman et al. 2008)

| | Baxter | | Biopure | | Hemosol | | Northfield | | Sangart | | Somatogen | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cohort | T | C | T | C | T | C | T | C | T | C | T | C |
| Number of subjects | 504 | 505 | 708 | 618 | 209 | 192 | 623 | 457 | 85 | 45 | 64 | 26 |
| 1. Death | 78 | 61 | 25 | 14 | 1 | 4 | 73 | 39 | 2 | 0 | * | * |
| 2. Hypertention | 76 | 38 | 166 | 59 | 113 | 75 | * | * | 7 | 1 | 8 | 0 |
| 3. Pulmonary Hypertension | 1 | 0 | 3 | 0 | * | * | * | * | * | * | * | * |
| 4. Chest pain/chest tightness | * | * | 21 | 16 | * | * | * | * | * | * | 6 | 0 |
| 5. Congestive heart failure | 0 | 1 | 54 | 22 | 0 | 2 | 17 | 20 | * | * | * | * |
| 6. Cardiac arrest | * | * | 17 | 6 | 1 | 1 | 14 | 9 | * | * | * | * |
| 7. Myocardial infarction | 6 | 1 | 14 | 4 | 14 | 7 | 29 | 4 | 2 | 0 | * | * |
| 8. Cardiac arrhythmias/ conduction abnormalities | 23 | 17 | 153 | 100 | 1 | 1 | * | * | 15 | 5 | 1 | 1 |
| 9. Cerebrovascular accident, cerebrovascular ischemia, TIA | * | * | 16 | 3 | 2 | 1 | 3 | 1 | * | * | * | * |
| 10. Pneumonia | * | * | 35 | 22 | * | * | 27 | 21 | * | * | * | * |
| 11. Respiratory distress/failure | * | * | 22 | 12 | * | * | 21 | 17 | * | * | * | * |
| 12. Acute renal failure | 1 | 3 | 10 | 4 | 2 | 2 | * | * | * | * | * | * |
| 13. Hypoxia, cyanosis, decreased oxygen saturation | * | * | 76 | 35 | 1 | 1 | * | * | * | * | 3 | 1 |
| 14. Hypovolemia | * | * | 19 | 4 | | | * | * | * | * | * | * |
| 15. Gastrointestinal | 51 | 31 | 645 | 195 | 23 | 1 | * | * | | | 36 | 6 |
| 16. Liver, LFTs abnormal | 27 | 8 | 20 | 5 | 8 | 0 | * | * | 57 | 20 | 6 | 3 |
| 17. Pancreatitis | 11 | 0 | 5 | 3 | 1 | 0 | * | * | * | * | * | * |
| 18. Coagulation defect, thrombocytopenia, thrombosis | * | * | 45 | 17 | 1 | 0 | 13 | 4 | * | * | * | * |
| 19. Hemorrhage/bleeding/ anemia | 33 | 22 | 108 | 55 | 1 | 1 | 20 | 17 | * | * | * | * |
| 20. Sepsis, septic shock, MOF | 2 | 2 | 15 | 6 | 0 | 1 | 26 | 20 | * | * | * | * |
| 21. Pancreatic enzyme inc | 13 | 4 | 3 | 0 | * | * | * | * | * | * | * | * |
| 22. Lipase increase | 29 | 9 | 48 | 12 | 19 | 2 | * | * | 8 | 4 | 7 | 1 |
| 23. Amylase increase | 48 | 45 | * | * | 35 | 20 | * | * | 7 | 2 | 4 | 1 |

*T* HBOC treated group
*C* Control solution treated group
* No information available
*Note* Apex and Enzon also conducted clinical trials but data were not reported

death in recent HBOC clinical trials (Silverman 2009, Table 39.1) and a highly controversial Meta analysis that HBOCs are associated with increased risk of MI and death (Natanson 2008) are hampering further development of HBOCs as viable therapeutics. Recent workshops organized by NIH and FDA (Estep et al. 2008; Silverman 2009; NIH 2011) discussed current issues and provided recommendations on directions of future HBOC research and development.

The causality of HBOCs for the observed SAEs has not definitively been established. To elucidate the pathophysiologic mechanisms of AEs observed with HBOCs, it is essential to understand how HBOCs affect key organ systems and their physiologic functions not simply in normal subjects but in patients. Studies conducted in models of healthy young animals have failed to predict the pathophysiologic responses observed in actual patients who often are older and present with multiple co-morbid conditions (e.g., diabetes, hypertension, cardiovascular diseases, etc.). It is essential that preclinical safety studies be conducted in animal models that closely simulate target patient conditions. To further the development, investigations are required to establish causality of the observed serious adverse events (SAEs) and to test HBOC used and determine the pathophysiologic mechanism involved. Only when armed with accurate knowledge of pathophysiologic mechanisms of adverse events (AEs), may appropriate modifications be made to the current HBOC products or develop a new generation of safer products.

More recently, Mozzarelli (2011) presented a more open view on the strategies for designing a new generation of safer and effective products. Certain HBOCs are also being developed as oxygen therapeutics (OTs) targeted for treatments of ischemic tissues and organs (e.g., ischemic heart/limb, ischemic stroke). In addition, because many civilian and military hemorrhagic trauma victims are presented with coagulopathy, MRFs that contain procoagulation agents are also in development.

## 39.3 Current Issues and Barriers

The current impediment in the progress of HBOC development is in large part due to insufficient scientific understanding of some critical mechanisms. How an individual HBOC formulation, when administered intravenously, interacts with the host mechanisms in heightened or compromised state by disease, surgery or traumatic injury especially presented with concurrent underlying co-morbid conditions (e.g., hypertension, diabetes, cardiovascular diseases). It is an extremely complex dynamic process involving multiple cellular, tissue and organ systems which are ultimately integrated into the whole systemic response and its fate. To help facilitate HBOC development, a NIH-NHLBI organized working group workshops in 2006 (Estep et al. 2008) and 2011 (NIH 2011) and identified some of the key issues holding up the progress of the field (Table 39.2) and made a series of recommendations (Table 39.3). In addition, there are some inherent limitations in a traditional industry-centered collaboration model (Kim 2011).

Some of the key issues are:

- Animal models did not predict adverse effects observed in clinical trials Results of most preclinical animal studies conducted with various candidate HBOC products were generally positive. However, preclinical animal models did not predict the AEs observed in clinical studies. Therefore, there is a need to identify/develop animal models that are relevant to target patient conditions and

**Table 39.2** Important issues to be addressed in HBOC development (Estep et al. 2008; NIH 2011)

- Development of animal models that more closely simulate human clinical conditions with co-morbidities (e.g., diabetes, hypertension, cardiovascular diseases)
- Development of new improved HBOCs (e.g., HBOCs with reduced vasoactivity and oxidative reactions, enhanced circulation time and shelf life)
- Investigation of mechanisms of cardiovascular and cerebrovascular events observed after blood substitutes infusion
- Significance of cardiac lesions observed after HBOC infusion
- Role of reactive oxygen species (ROS) in the etiology of human clinical side effects
- Exploration of interactive effects between blood substitutes infusion and concurrent fluid, drug and anesthetic therapies
- Effect of concurrent stress, particularly local or systemic inflammation, in response to blood substitutes infusion
- Investigation of the cause of bradycardia associated with HBOC administration
- Further study of mechanism and clinical significance of vasoactive effects of HBOCs
- Evaluation of the mechanism and clinical significance of gastrointestinal distress, pancreatic toxicity and liver and pancreatic enzyme elevation
- Comparative assessment of the antigenic and immunomodulatory properties of different blood substitutes
- Effects of formulation excipients
- Further study of the distribution and metabolism of HBOCs

**Table 39.3** 2006 NIH workshop recommendations (Estep et al. 2008)

- Exploration of the mechanism(s) of adverse side effects that have been observed during the clinical testing of HBOC formulations. Particular priority should be given to the investigation of cardiovascular and cerebrovascular events. The use of animal models with compromised cardiovascular systems and/or altered physiology in the evaluation of HBOC solutions is highly encouraged
- Further evaluation of the distribution and metabolism of different Hb derivatives, especially with reference to the role that these factors play in the etiology of adverse events and the determination of functional intravascular persistence
- Continued research into the physiology of oxygen delivery by acellular formulations ranging from subcellular to global levels of response, with emphasis on the microcirculation in different tissue beds
- Assessment of whether enhanced generation of ROS after HBOC infusion is responsible for clinically observed adverse events in humans
- Evaluation of the impact of HBOC formulation excipients on product toxicity and stability
- Development of new Hb active entities with improved adverse event profiles and enhanced intravascular functional persistence
- Identification and use of improved models for the comparative assessment of HBOC formulation safety and efficacy. Such models should be predictive of response in humans, incorporate stress conditions and be used to systematically evaluate the effect of variation in Hb structure, biochemistry and physical chemical properties
- Production and distribution of highly purified HBOC solution(s) in sufficient quantity to support the research and testing advocated elsewhere in this report
- Comprehensive assessment and reporting of the adverse events and physiologic response of HBOC solutions evaluated in clinical trials, recognizing that such an assessment would require the permission of commercial manufacturers and collaboration with the US FDA
- Development and validation of a noninvasive method for the routine clinical assessment of critical organ oxygenation to better inform decisions to transfuse HBOCs and blood

to employ more sensitive tests that could detect molecular and cellular dysfunction as well as organ-specific toxicities and systemic abnormalities.

- Limited availability of test materials (HBOCs/OTs)
  One well recognized issue in HBOC development is limited availability of test HBOC solutions for conducting independent investigations. Because most HBOC products are not yet marketed, independent investigators have difficulty obtaining test HBOC products to conduct evaluations. To allow more independent evaluations of candidate HBOC products, 2006 NIH-NHLBI Workshop (Estep et al. 2008) recommended that supply of sufficient amount of well characterized standardized HBOC formulations be made available to the general research community. To help accomplish that recommendation, NIH awarded a SBIR contract to a company that now make its products available for investigators albeit at a cost. However, because AEs and toxicities have been observed with different HBOC products, it is important that more than one product be made available to investigators. In addition, as deemed necessary certain experimental (as well as marketed) procoagulation products (e.g., platelets, fresh frozen plasma, cryoprecipitate, rFVIIa anti-fibrinolytics) should also be made available for development of MRFs. Therefore, this consortium will invite producers of HBOC/OT/MRF products to participate knowing that this will be a NIH/FDA guided pathway for possible regulatory approval. As such, the FDA will be invited in these deliberations and expected to provide advice/guidance to create a validated pathway for potential approval of products.
- Causality of HBOC in observed SAEs
  Cause(s) of the SAEs observed in HBOC clinical trials have not been thoroughly investigated. This is in part due to lack of detailed and objective information/data regarding the nature/circumstances of observed AEs. Currently, major HBOC developmental efforts are led by few companies that adopt a traditional industry-centered research model. This approach is a largely a 'closed' system in which most data (especially negative data) are kept confidential among the close collaborators only. The exact nature of negative results is not generally made available to a wider group of independent unbiased investigators. Therefore, outside researcher are often deprived of the opportunity to the timely investigation of SAEs and other side effects/toxicities. This 'closed' approach significantly hampers expeditious development of possible resolutions.

## 39.4 The HBOC/OT/MRF Research Consortium, a Way Forward

To facilitate progress of HBOC development, we propose a HBOC research consortium of key leading academic investigators and select HBOC producers from US, Europe and Asia. The consortium will serve as a think tank and a

coordinating body for collaborative efforts in investigation of the key unresolved scientific issues that are hampering further progress in HBOC development. The HBOC research consortium will facilitate development of viable HBOC products through concerted efforts of the some of the world's leading experts in the field. To encourage constructive discussions/solutions for key unresolved issues, the consortium will adopt an open communication policy and objective and transparent processes in the conduct of research. The goal of the consortium is to foster orchestrated collaborations and constructive discourse and to breakdown barriers that impede development of viable HBOC products.

Some key goals of the consortium are:

- Foster collaboration for expeditious resolutions of key unresolved issues in HBOC development.
- Coordinate collaboration to minimize unnecessary duplications/redundancies for maximum efficiency and conserve resources.
- More objective and transparent evaluation of candidate products by independent investigators exploiting state of the art methods to investigate physiological and biochemical mechanisms.
- Facilitate information/data exchange and prompt and timely dissemination of research findings through open presentations, publications and other media.
- Serve as a central 'library' for HBOC research and other relevant information obtained from public sources or voluntarily provided by authors, study sponsors and publishers. (copy right issue will be openly discussed and negotiated).
- Identify and secure funding for HBOC research/development (e.g., national and international, public and private funding agencies).
- Foster young investigators to enter into the field.
- Identify an optimal HBOC formulation and/or develop a new viable product in the next 10 years, including basic science, translational and FDA approved Phase 1 clinical trials.
- FDA will be invited to participate from the ground level to provide advice in design of experiments, protocol development and formulating guidelines to ensure that required preclinical and clinical studies are performed according to Good Laboratory Practice (GLP) and Good Clinical Practice (GCP) and other regulatory guidelines. This FDA guided product development approach will serve as a 'validated' pathway to eventual product approval.

To achieve the stated goals, the consortium will utilize a multi-disciplinary approach. The core groups of the consortium will be US-based but to maximize 'brain power' for more expeditious development, a select group of leading international experts will also be included (see Table 39.4). In addition, to maximize the probability of successful outcome (discovery/identification of a successful product), the consortium will evaluate multiple candidate products with distinct characteristics. The candidate HBOC products will be studied (for selected projects) by member investigators with no conflict-of-interest issues with the products being tested. All investigators agree to participate in the consortium will be asked to disclose any conflict-of-interest issues. If found a clear conflict, he/she

**Table 39.4** Some potential projects (investigators will be invited based on relevant expertise)

- Systems biologic approach to investigation of HBOC-mediated AEs (e.g., hypertension/vasoconstriction, enzyme abnormalities, cardiac abnormalities, etc.) utilizing molecular, genomic and proteomic analytical tools as HBOC interaction with cells/organs is dynamic multi-faceted process necessitating collaborative efforts of multi-disciplinary experts
- Robust global safety evaluation of HBOCs/OTs/MFRs via total body assessment based on organ proteomics and clinical assays
- Temporal and between group comparison of key physiological parameters before, during and after infusion of control and test HBOC/OT/MRF agents using in vivo analytical tools including single photon emission computed tomography (SPECT)
- Role of HBOCs in radical mediated toxicity and organ dysfunction in hemorrhagic shock/resuscitation
- Mechanism(s) of HBOC-mediated vasoconstriction/hypertension and it relationship to observed AEs and organ dysfunction
- Toxicities or harmful interactions between HBOCs and a patient's underlying disease
- Study of mechanisms of cell-free HBOC-mediated AEs/SAEs
- Role of vascular endothelial dysfunction (including NO and endothelin response and barrier function)/inflammation on physiological response to HBOCs
- Pathophysiologic relationship of post-trauma/hemorrhage immunosuppressive conditions and HBOC-mediated AEs
- Development of MFRs that include a crystalloid solution, oxygen carrier and procoagulant agents
- Others deemed necessary and appropriate

will be withdrawn from participation or conduct of certain studies. Qualifying investigators will submit a specific research proposal studying a selected HBOC/OT/MRF product(s) according to a format adopted by the consortium in consideration of potential funding sources (including a full budget proposal within a proposed direct cost cap). Most relevant high priority projects/investigators will be selected and included in the final consortium research proposal to be submitted to an appropriate funding agency. Ethics Committee, Data Safety Monitoring Board and strict adherence to local Institutional Review Board policies will be enforced as appropriate.

### *39.4.1 Proposed Activities of Consortium*

- Coordination of collaboration in a concerted manner to bring about investigation with efficient use of resources.
- Identify and define highest priority issues/areas to resolve in HBOC/OT/MRF research/development for the consortium investigator to undertake.
- Evaluation of several distinct multi-product candidates (e.g., acellular and cellular HBOCs, OTs, MRFs, etc.).

- Data mining of literature (and possibly relevant FDA database if proper arrangement can be made) for in-depth analyses utilizing system's biology approach.
- Identify and develop avenues/means to undertake collaborative research including possible source of funding.
- Data/information exchange/workshop on focused topics.
- Identify and develop standardized methods/assays/tools/test HBOCs and specialty reagents and quality standards for preclinical and clinical tests.
- Repository for test HBOCs/OTs/MRFs, preclinical and clinical study data, relevant literature and regulatory information/advice.
- Others as deemed appropriate.

Specific terms of collaborative activities including nature of projects, execution of experiments, data management/dissemination, copyright/IP and other issues will be defined in a written Memorandum of Understanding (MOU).

### *39.4.2 Organizing Members*

- Hae Won Kim, Ph.D., Brown University, Providence, RI, USA.
- Jonathan S. Jahr, MD, UCLA, Los Angeles, CA, USA.
- Andrea Mozzarelli, Ph.D., University of Parma, Parma, Italy.
- Hiromi Sakai, Ph.D., Department of Chemistry, Nara Medical University, Kashihara, Japan.

The core of the consortium will be U.S.-based. Dr. Hae Won Kim (Brown University, Providence, RI) and Dr. Jonathan Jahr (UCLA, Los Angeles, CA) will serve as co-Directors and share responsibilities in the overall management and coordination of consortium activities. Additional members with expertise in selected areas of interest will be included once priority areas/projects are determined (see Table 39.4, for potential projects). Specific roles and responsibilities of each consortium member will be defined in a written MOU agreement.

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