Chapter 33
HBOCs and Cardiac Integrity

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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 -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 -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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