Chapter 32
Acellular Hemoglobin-Based Oxygen Carrier Mediated Blood Pressure Elevation and Vasoconstriction: A Review of Proposed Mechanisms and Contributing Factors

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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

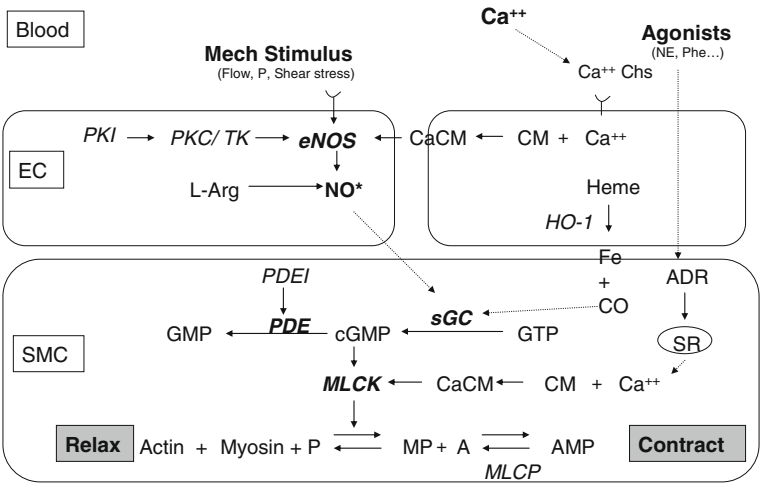

Fig. 32.1 Role of endothelial NO in the vascular smooth muscle contraction/relaxation. EC: endothelial cell, SMC: vascular smooth muscle cell, AchR: acetylcholine receptor, Ca^{++}Chs: calcium channels, PKI: protein kinase inhibitor, PKC: protein kinase C, TK: tyrosine kinase, CM: calmodulin, PDE: phosphodiesterase, 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 β-globins (β93Cys) 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 β 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 β-β 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(μ) would increase blood flow (Q) by decreasing the vascular resistance assuming vascular radius (r), driving pressure (ΔP) 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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