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VASOACTIVE PROPERTIES OF SYNTHETIC BLOOD SUBSTITUTES

LUIZ F. POLI DE FIGUEIREDO

Department of Cardiopneumonology, Faculty of Medicine, Universidade de São Paulo and Department of Surgery, Universidade Federal de São Paulo, Brazil

Abstract There is a great need for the development of a safe and efficient blood substitute, to overcome the important limitations of homologous blood transfusion. Currently available cell-free hemoglobin-based oxygen-carrying solutions present oxygen transport and exchange properties similar to blood and potential benefits over conventional transfusion, including large supply, absence of transfusion reactions, no need for cross-matching, no risk for transmission of disease and long shelf life. Several experimental studies have suggested that cell-free hemoglobin is a vasoactive agent. In animal models of hemorrhagic shock, small doses of cell-free modified hemoglobin restore arterial pressure, promote adequate tissue oxygenation, and improve survival, when compared with fluids with no oxygen-carrying capacity. On the other hand, it has been demonstrated that hemoglobin-induced vasoconstriction may result in decreased cardiac output, reduced blood flow to vital organs and severe pulmonary hypertension. Cell-free hemoglobin solutions cause their pressor effects by binding and scavenging nitric oxide. Although hemoglobin within the red blood cells is the natural scavenger of NO, when the hemoglobin is free in solution, NO is inactivated to a greater extend. Cell-free hemoglobins are on advanced clinical trials, despite the fact that several concerns raised by experimental studies have not been adequately addressed in early clinical trials. The development of a safe and efficient blood substitute depends on the availability of these products for critical evaluation by the scientific community before the widespread clinical use of these blood substitutes.

Resumen ***Propiedades vasoactivas de sustitutos sintéticos de la sangre.*** Hay una urgente necesidad de desarrollar un sustituto de la sangre con el fin de resolver las importantes limitaciones de las transfusiones de sangre homóloga. Las soluciones que aportan oxígeno a través de hemoglobina libre de células ofrecen un transporte de oxígeno e intercambio similares a la sangre con beneficios potenciales, frente a la transfusión convencional, que incluyen un rápido suministro, ausencia de reacciones transfusionales sin requerimiento de cross-matching, ningún riesgo de transmisión de enfermedad y una larga vida en el stock. Varios estudios experimentales sugieren que la hemoglobina libre de células es un agente vasoactivo. En modelos animales de shock hemorrágico, pequeñas dosis de hemoglobina acelular restablecen la presión arterial, proveen adecuada oxigenación tisular y aumentan la sobrevida, en comparación con fluidos sin ninguna capacidad de proveer oxígeno. Por otro lado, se ha demostrado que la vasoconstricción inducida por la hemoglobina puede provocar una disminución en el volumen minuto, una disminución en el flujo sanguíneo hacia órganos vitales y severa hipertensión pulmonar. Soluciones de hemoglobina acelular inducen sus efectos presores ya sea por unión o secuestro de óxido nítrico (NO). A pesar de que la hemoglobina dentro de los eritrocitos es el natural depurador de NO, la hemoglobina libre en solución inactiva NO aun más. Hay ensayos clínicos en curso con hemoglobina acelular a pesar de que todavía no se han resuelto algunos de los problemas que surgieron en estudios previos. El desarrollo de un sustituto de la sangre seguro y eficiente depende de la disponibilidad de estos productos para una evaluación crítica por parte de la comunidad científica antes de su distribución clínica.

Key words: blood substitutes, hemoglobin, shock, nitric oxide, vasoconstriction

Blood performs a great variety of important physiologic tasks, but its most basic and critical function is to provide a continuous supply of oxygen to the tissues. Since oxygen is poorly soluble in plasma, the hemoglobin within the red blood cells (RBCs) is responsible for the transport of more than 98% of this gas. Cell-free hemoglobin solutions are the older and most studied "blood substitutes" or, more accurately defined, "oxygen-carrying volume expanders", since these solutions do not have coagulant, immunological and other functions that are performed by blood.

Homologous RBCs transfusion is currently safe, very efficient and, most importantly, has been widely tested, usually in the most critically ill patients, in very large doses, and in a great variety of clinical settings. Moreover, the majority of the transfused RBCs can survive for weeks or months and presents oxygen-carrying capacity and

Postal Address: Dr. Luiz F. Poli de Figueiredo, Instituto do Coração, Fac. Medicina, Universidade de São Paulo, Av. Enéas de Carvalho Aguiar, 44, São Paulo - SP 05403-000, Brasil
FAX: 55-11-853.7887; E-mail: expluiz@incor.usp.br

elimination characteristics similar to native blood. For all these reasons, RBCs transfusion is considered the "gold standard" to which safety and efficacy of any other oxygen-carrying solution should be compared[1].

Despite the remarkable safety record for homologous RBCs transfusions, they have very important limitations, including a major medical and public concern with transmission of diseases like HIV, hepatitis, and other bacterial, parasitic and viral blood-borne diseases[2-4]. Additionally, there is a progressive increase in blood demand, while blood supply may be critically decreased by the aging population and inadequate rates of volunteer donations in the near future[5]. There are other limitations, inherent to the current techniques employed for RBCs transfusions, such as the requirement for compatibility testing, the risks for transfusion reactions and human errors, the short shelf life, limited to weeks, and the rigid storage requirements[1, 6].

These are the major reasons why there is a great need for the development of a safe and efficient oxygen-carrying solution that could replace RBCs transfusions. Cell-free hemoglobin-based oxygen-carrying solutions that are currently available present oxygen transport and exchange properties similar to blood and potential benefits over conventional transfusion, including large supply, absence of transfusion reactions, no need for cross-matching, no risk for transmission of disease and long shelf life, up to one year if frozen. They represent substantial improvement over the earlier hemoglobin solutions, which caused marked toxicity and severe side effects. Several highly purified, chemically modified hemoglobin-based oxygen carriers are now undergoing clinical trials[7, 8]. However, there is evidence that cell-free hemoglobin is a vasoactive agent. The aim of this report is to discuss the vasoactive properties of the hemoglobin-based oxygen carriers.

Effects of the earlier hemoglobin solutions

Earlier hemoglobin solutions were produced using RBCs that were hemolyzed with distilled water, and made isotonic by adding salt[9]. Oxygen transport and life were preserved in animal models of complete exchange transfusion, in otherwise lethally low hematocrits[1, 6, 10]. In small human trials, these solutions caused several reactions including fever, nausea, vomiting, hypertension, bradycardia, bleeding, intravascular coagulation, and marked oliguria. On the other hand, some patients in shock showed restoration of arterial pressure and improved mentation with small amount of hemoglobin solution[11]. Improvement in the preparation resulted in hemoglobin solutions without cell membrane residues, the stroma-free hemoglobin (SFH). However, undesirable side effects were evident in a well conducted clinical trial, which had shown that small doses of SFH, to healthy normal volunteers, caused transient hypertension, bradycardia, oliguria and gross hemoglobinuria[12]. This study proved that unmodified human hemoglobin was toxic and highlighted the critical importance of preventing the glomerular filtration of the dissociated hemoglobin tetrameter that, precipitating in the proximal tubule, caused renal damage[1, 13]. Moreover, SFH presents a very short half life, because hemoglobin dimers and monomers are quickly excreted by the kidneys, and also a high oxygen affinity, caused by the loss of the effects of 2,3-diphosphoglycerate, limiting oxygen unloading at the tissues[1, 13].

Modified cell-free hemoglobins

Chemical modifications of SFH solutions decreased oxygen affinity, prolonged half-life and prevented renal damage, resulting in the actual oxygen carrying hemoglobin-based blood substitutes that are being tested clinically. All these modified hemoglobins are highly purified, free of phospholipids, endotoxins, viral and bacterial contaminants. Several different methods were employed, including pyridoxylation, polymerization, conjugation, encapsulation, intramolecullar crosslinking, the production of recombinant hemoglobin and the use of bovine hemoglobin, which does not require 2,3-DPG and has an oxygen affinity similar to human hemoglobin [1, 6, 9, 14]. These modifications resulted in hemoglobin solutions with P_{50} (oxygen partial pressure resulting in a 50% hemoglobin saturation) values similar to native blood, while half-life was prolonged to up to 36 hours and renal toxicity was decreased by the maintenance of the tetrametric structure, reducing the rapid clearance of hemoglobin diamers by the kidneys[1, 6, 13].

Experimental studies with modified hemoglobins

These solutions have been widely tested in animal models of hemorrhagic shock and whole blood exchange, which demonstrated maintenance of cardiovascular function and oxygen metabolism, and long-term survival after partial and complete exchange transfusion[15-20].

Vasoactivity of these modified hemoglobin solutions remained striking, particularly in animal models of hemorrhagic shock, in which even small doses of cell-free modified hemoglobin restored arterial pressure, promoted adequate tissue oxygenation and improved survival, when compared with fluids with no oxygen-carrying capacity[21-26]. It has been suggested that these solutions, because of their pharmacological actions and unique pressor-perfusion effects of increased arterial

pressure, cardiac output and organ blood flows[27-35], offer particular potential as a resuscitative fluid for trauma and hemorrhagic shock. However, we and others have demonstrated that hemoglobin-induced vasoconstriction resulted in decreased cardiac output and reduced blood flow to the intestines, kidneys and heart, using animal models of hemorrhagic shock, hemodilution, sepsis and isolated organs[36-44]. The vasopressor effect is even more pronounced in the pulmonary vasculature, causing severe pulmonary hypertension, that can lead to hemodynamic instability and acute right ventricular dysfunction[38-40, 43, 44].

Figure 1 illustrates the vasoactive properties of one of the most widely tested hemoglobin solutions, the alpha-alpha cross-linked hemoglobin (aaHb), given to hemorrhaged pigs, which received a 2-min, 4 ml/kg bolus injection of either aaHb or an oncotically matched 7% human albumin solution (ALBh) as the only treatment[43]. This amount of fluid was equivalent to only one fourth of the shed blood to maintain mean arterial pressure around 40 mmHg for 60 minutes. We can see the immediate and sustained arterial pressure improvement after aaHb, which was achieved only through vasoconstriction, with no improvement in cardiac output. Pulmonary arterial pressure peaked immediately after aaHb, and remained above baseline throughout the experiment.

Although some degree of systemic vasoconstriction may be desirable for the initial resuscitation of hypovolemic shock, by restoring coronary and cerebral perfusion pressures and brain blood flow[43], pulmonary hypertension and coronary vasoconstriction are highly undesirable side effects, with potential for catastrophic hemodynamic events, particularly in patients with the greatest need for blood substitutes, such as the ones undergoing trauma and major cardiovascular and cancer operations. Sustained vasoconstriction may also affect renal perfusion and, although evidence of long term renal damage has not been reported with these modified hemoglobins, most studies have been performed in normal animals, with preserved organ functional reserve before undergoing the acute insult. It is largely unknown the impact that hemoglobin-induced vasoconstriction may produce in the kidneys and other organs acutely or chronically compromised. Impairment of functional capillary density, after hemodilution with modified hemoglobin solution, has been demonstrated in a videomicroscopy study of hamsters microcirculation, when compared with non-oxygen-carrying colloids[45]. It has not been established whether this is mainly caused by vasoconstriction or because modified hemoglobin carries too much oxygen, eliciting a metabolic autoregulatory effect[45]. However, direct measurement of tissue oxygen content has suggested that tissue oxygenation with cell-free hemoglobin is reduced compared to RBCs[46].

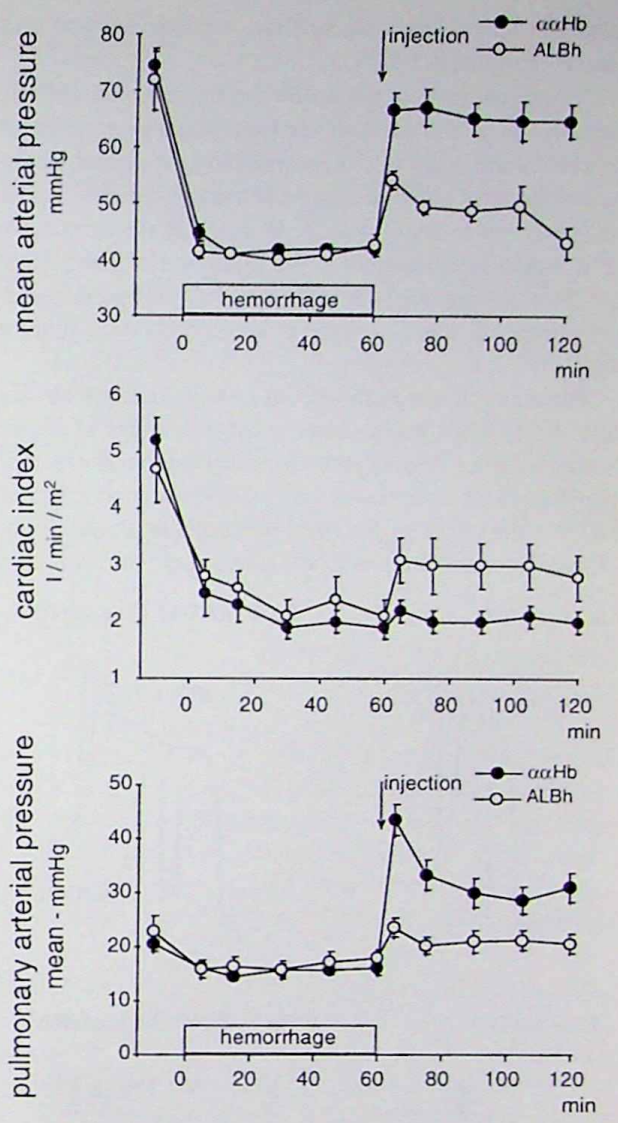

Fig. 1.- Hemodynamic responses to a 4 ml/kg, 2-min bolus injection of either 10% aaHb (n=7) or 7% human serum albumin (ALBh, n=7) to hemorrhaged pigs (shed blood = 17±1.2 ml/kg).[43]

Mechanism for cell-free hemoglobin vasoactivity

There is large evidence that cell-free hemoglobin solutions produce their pressor effects by binding and scavenging nitric oxide (NO), the potent endothelium-derived vasodilator responsible for the normal vasodilatory tone in the systemic and pulmonary circulation[1, 6, 9, 35-38, 42, 47-51]. Some authors have suggested that endothelin release and other vasoconstrictors have also play a role[34, 52]. When the hemoglobin is within the RBCs, NO is removed as it dissolves into the plasma and ultimately interacts with hemoglobin. However, when the hemoglobin is free in solution, NO is inactivated to a greater extend, thereby causing vasoconstriction. Free-hemoglobin binds NO

thousand times more avidly than it binds oxygen and carbon monoxide[6, 53].

The vasoactivity of the aaHb can be demonstrated by the tracings of representative experiments performed on isolated blood vessels[54], demonstrating the endothelium-dependence of aaHb-induced contraction (Figure 2), the endothelium-independence of sodium nitroprusside (SNP)-induced relaxation in the presence of aaHb (Figure 3) and time-dependent and endothelium-independent effects of aaHb on SNP-induced relaxation (Figure 4)[54].

Because of this high affinity of free hemoglobin for NO, it has been suggested for the treatment of septic shock, in which hypotension and low peripheral vascular resistance are associated with excessive production of NO[35, 38]. Given to septic rats, hemoglobin solutions increased arterial pressure, improved regional perfusion to vital organs and improved mortality[35]. Treatment with hemoglobin also improved arterial pressure without a significant impairment in blood flow to the kidneys and in-

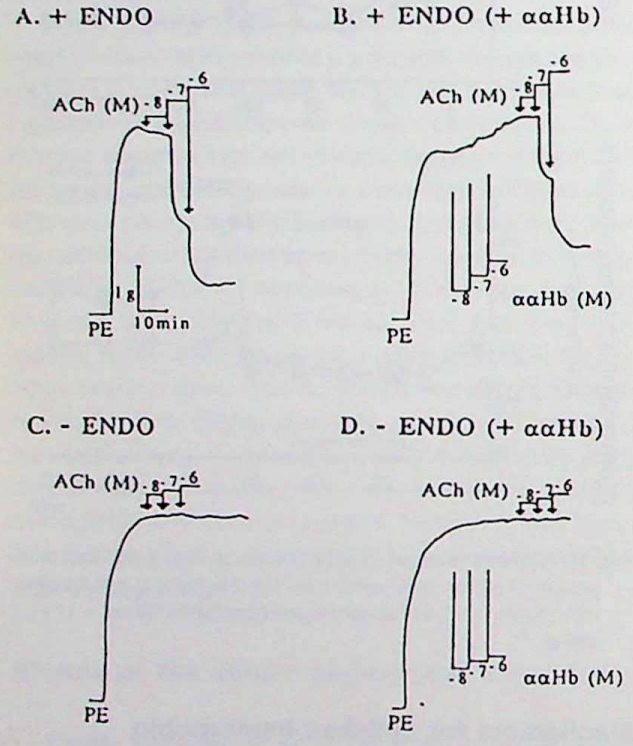

Fig. 2.- Endothelium-dependence of aaHb-induced contraction. Representative tracings showing the contractile response of rat aortic rings, precontracted with phenylephrine (PE, 10^{-3} M), exposed to increasing concentrations of aaHb (1,8 x 10^{-8} to 1,8 x 10^{-5} M) with and without endothelium. Endothelial removal was produced by rubbing gently the internal surface of the rings with two wires. Tissue viability was assured by the adequate contractile response to phenylephrine (10^{-3} M). The presence of endothelium (+ENDO) was established by the relaxation response to acetylcholine (ACh, 10^{-8}, 10^{-7}, and10^{-6} M), while the absence of endothelium (-ENDO) was confirmed by the absence of relaxation in response to ACh. In the presence of endothelium, aaHb presented a concentration-dependent contractile response (B.+ENDO,+aaHb). In addition, there was a decreased ACh-induced relaxation in the presence of aaHb. Without endothelium, there was no contractile response to aaHb (D.-ENDO,+aaHb).[54]

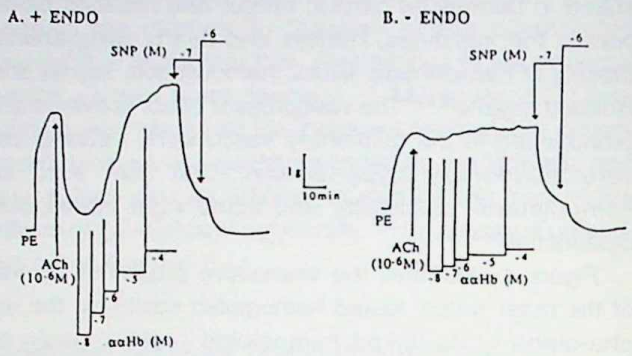

Fig. 3.- Endothelium-independence of SNP-induced relaxation in the presence of aaHb. Representative tracings showing the contractile response of rat aortic rings, precontracted with phenylephrine (PE, 10^{-3} M), after ACh (10^{-6} M) and aaHb (1,8 x 10^{-8} to 1,8 x 10^{-4} M), with and without endothelium. Experimental setup was described on figure 2. ACh caused marked relaxation in the presence of endothelium and the addition of aaHb produced concentration-dependent contractile response, reversing completely ACh-induced relaxation. In the absence of endothelium, there was no ACh-induced relaxation and no aaHb-induced contraction. Sodium nitroprusside (SNP 10^{-7} and 10^{-6} M), an NO donor, caused relaxation with and without endothelium. Vasodilation induced by NO donors is independent of endothelium and reverses aaHb-induced contraction.[54]

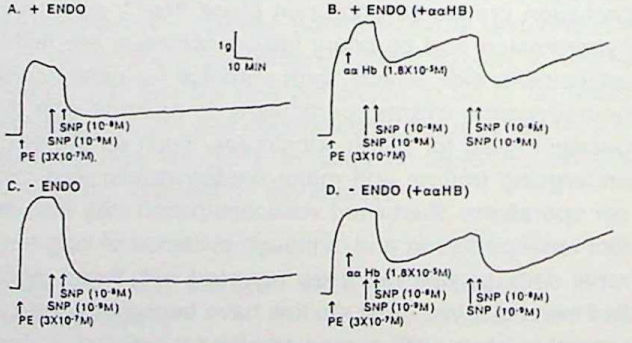

Fig. 4.- Time-dependent and endothelium-independent effects of aaHb on SNP-induced relaxation. Representative tracings showing the contractile response of rat aortic rings, precontracted with phenylephrine (PE, 10^{-3} M), in which the relaxation response to sodium nitroprusside (SNP, 10^{-9} e 10^{-8} M) was established with (+ENDO) and without endothelium (-ENDO). Experimental setup was described on Figure 2. SNP (10^{-7}) induced marked and sustained relaxation with and without endothelium (A.+ENDO, C.-ENDO). aaHb (1,8 x 10^{-5}), produced contraction only in the presence of endothelium (B.+ENDO, +aaHb). The addition of SNP, 10^{-9} M, caused minimum relaxation while SNP, 10^{-8} M, caused a partial and transient relaxation, with and without endothelium. The repetition of SNP (SNP, 10^{-9} and 10^{-8} M) produced similar partial and transient relaxation. These data suggest that, in the presence of aaHb, NO donors will be required in higher doses and for longer periods if they are to be used clinically to overcome hemoglobin-induced vasoconstriction.[54]

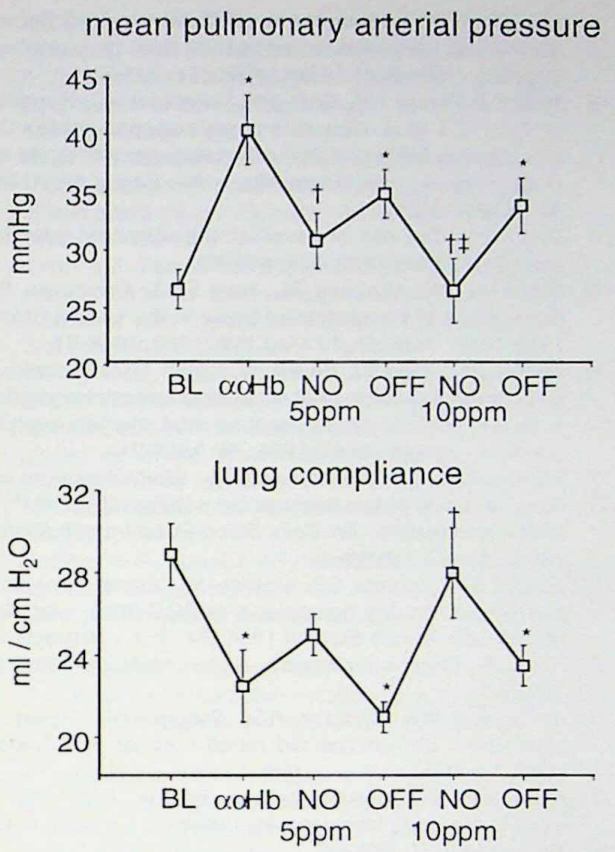

Fig. 5.- Effects of 2-ml/kg of 10% aaHb, infused over 20 minutes to normovolemic, phentanyl-anesthetized pigs (n=5), on pulmonary arterial pressure and lung compliance. aaHb produced marked increases in pulmonary arterial pressure and decreases in lung compliance. Inhaled nitric oxide (NO 5 ppm and 10 ppm), in 10-min cycles,, reversed pulmonary hypertension and decreased lung compliance.[56]

testines in endotoxemic pigs; however, hemoglobin caused a significant exacerbation of endotoxin-induced pulmonary hypertension and arterial hypoxemia[38]. Hypoxemia, ventilation-perfusion abnormalities and greater acidosis were also observed with cell-free hemoglobin infusion in a model of canine bacteremia[55]. We were able to selectively reverse aaHb-induced pulmonary hypertension and decreased lung compliance with small doses of inhaled nitric oxide (Figure 5)[56].

Other potential problems with cell-free hemoglobin

Besides these undesirable hemodynamic and ventilatory effects, there are other potential problems with the use of cell-free hemoglobin for sepsis. Hemoglobin may bind the individual molecules of lypopolysaccaride, breaking up the endotoxin mycelles and increasing the biologic activity of bacterial endotoxin[57,58]. Iron and heme may cause bacteria to grow, worsening infections[58]. White cells and platelets activation has been shown with cell-free hemoglobins, which could promote the release of several proinflammatory cytokines and procoagulants[59-61].

Two important enzymes normally present within the RBCs, superoxide dismutase and catalase, are able to remove oxygen radicals and peroxides, and they are absent in cell-free hemoglobin solutions. Hemoglobin breakdown products, the heme and iron, participate in redox reactions and are capable of accelerating the generation of oxyradicals by the Fenton and Haber-Weiss reactions, with potential for increased lipid peroxidation and other forms of cell damage related to reperfusion injury[9,62,63]. Iron clearance mechanisms may became rapidly saturated with the rates of iron loss from the cell-free hemoglobins[64]. Methemoglobin levels can be greatly increased by cell-free hemoglobin, since a critical step for superoxide production and for hemoglobin breakdown is the rate of methemoglobin formation, and it does not carry oxygen[40].

Other pharmacological disadvantage of cell-free hemoglobins include a shorter intravascular half-live, from 8 to 36 hours[1]; they also have colloid osmotic activity and changes in intravascular volume could be expected as redistribution and clearance occurs. With cell-free hemoglobin diffusion into the interstitial space, intravascular concentration declines and extravascular concentration increases, leading to intravascular fluid loss[1,9,13,65]. This fact, in addition to hemoglobin-induced vasoconstriction and increased arterial pressure, may mask a hypovolemic state after the use of large volumes of hemoglobin. Modified hemoglobin solutions is scavenged primarily by the reticuloendothelial system and long term effects have not been established.[1,66]

Clinical experience with cell-free hemoglobins

Small doses of several cell-free hemoglobins were administrated to healthy volunteers or in healthy anesthetized patients[7,8], with no report of death, allergic reactions or major side effects. Although safety has been claimed, these studies, unfortunately, have not been published in the scientific literature, making a correlation between the concerns above discussed and the clinical records.

We know that some early phase I safety trials were temporarily halted by the FDA because of medical events including fever, flu-like symptoms, headache, abdominal pain, gastrointestinal symptoms, muscle aches, increased blood pressure, decreased heart rate, chest pain and abnormal blood chemistries[9,67]. Human trials in Guatemala in 1990 and in nine children with sickle cell anemia in Zaire showed no untoward side effects, but surprisingly little information is available in these full papers[9,14].

More recently, larger phase I and II clinical trials, including hundreds of patients, are being performed but limited data is available in abstract forms[7, 8]. Safety was evaluated in 130 hemorrhagic shock patients at ten sites in the United States and Europe with cell-free hemoglobin solution, but no other information about these trials is available[7].

In humans, the vasopressor effect with cell-free hemoglobin is evident even in very low doses. It has been suggested that the vasopressor effect could be beneficial for patients with hypotension during hemodyalisis[68] and for septic patients with low peripheral resistance[69]. In one study, the vasopressor effect was also evident in the pulmonary circulation, after a very low dose of cell-free hemoglobin[70].

Marked elevations in amylase and lipase levels with synthetic hemoglobin but with no clinical evidence of pancreatitis or other major problems were reported in two studies[71, 72]. In addition to increased levels of pancreatic enzymes, increased arterial pressure was observed in awake patients during preoperative normovolemic hemodilution, highlighting the marked vasopressor effect[72].

It is surprising that human studies evaluating safety do not appear to directly address the concerns raised in preclinical studies regarding the vasoconstriction affecting the systemic and, particularly, the pulmonary circulation, that could and should be easily evaluated with echocardiography or with invasive techniques. Just a month ago, through a Company Press Release on the Internet, we learned that Baxter Healthcare halted its phase III trauma trial on the use of Diaspirin αα-crosslinked hemoglobin, planned to include 850 patients, because after 100 patients, mortality was greater with the use of synthetic hemoglobin[73]. Surgical patients and critical care patients are ideal candidates for a more extensive evaluation of these blood substitutes, because if safety and efficacy are proven, they will likely benefit those patients with multiple coexisting diseases and limited organ reserves. In 1990, the journal *Science* addressing the incredible relation between blood, money and research stated that "Solutions of modified hemoglobin could replace whole blood in many transfusions if researchers can learn how to avoid their potentially dangerous side effects"[74]. Greater availability of these solutions for an independent scientific community is crucial for a faster development of a very much needed safe and efficacious blood substitute.

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In order that people be happy in their work, these three things are needed: They must be fit for it. They must not do too much of it. And they must have a sense of success in it.

Para encontrar felicidad en el trabajo se necesitan tres cosas: Debe tener aptitud para ese trabajo. No debe trabajar demasiado. Debe sentirse exitoso.

John Ruskin (1819-1900)