Artificial Blood

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Abstract

Elimination of unwanted side-effects, especially transfusion-transmitted diseases (HIV and hepatitis) and leucocyte-mediated allosensitisation, is an important goal of modern transfusion medicine. The problems and high cost factor involved in collecting and storing human blood and the pending world-wide shortages are the other driving forces contributing towards the development of blood substitutes. Two major areas of research in this endeavour are haemoglobin-based oxygen carriers (HBOCs) and perfluorochemicals. Even though they do not qualify as perfect red blood cell substitutes, these ‘oxygen carrying solutions’ have many potential clinical and non clinical usages. These can reach tissues more easily than normal red cells and can deliver oxygen directly. These are not without adverse effects, and extensive clinical trials are being conducted to test their safety and efficacy. New understandings on the mode of action of these products will help to define their utility and application. Only after successful clinical trials can they be used for patient management, after approval by the FDA.

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Key Words : Blood substitute; Haemoglobin solutions; Perfluorocarbons

Introduction

There is a quest going on for a suitable blood substitute, which can be stored on a shelf, and infused safely at any time and in any place, regardless of the blood type. Strictly speaking, infusion of any material other than autologous blood, is actually infusion of blood substitute. Thus, the history of blood transfusion can be regarded as the history of blood substitute. Substances like milk, casein derivatives, starch, saline and Ringer’s solution had been tried prior to the first successful human to human transfusion (Blundell, 1824). Since World War II, the search for a suitable alternative to blood has been intensified, to enable coping with war like situations and large-scale civilian disasters. The functions of each blood component and the substitution of each are quite familiar, excepting that of oxygen carrying capacity. There has been considerable progress in two major classes of substitutes viz. haemoglobin solutions and perfluorocarbon (PFC) emulsions [1].

Haemoglobin-based oxygen carriers (Fig 1)

Von Stark (1898) was the first to use haemoglobin (Hb) solution in the treatment of a patient suffering from anaemia. A lot of research has subsequently modified Hb solution to form purified Hb solutions. Currently, HBOCs represent an interesting class of blood substitutes, which are undergoing advanced clinical trials. The therapeutic goal of these compounds is to avoid or reduce blood transfusion in different surgical and medical situations of acute Hb deficiency. Their main advantages include availability in large volumes, storage for prolonged periods, rapid administration (without typing and cross matching) and sterilisation by pasteurisation. Their main known disadvantages are, reduced circulation half-life, haemodynamic and gastrointestinal perturbations, probably related to nitric oxide (NO) scavenging, free radical induction, and alterations of biochemical and haematological parameters (increase in liver enzyme levels, platelet aggregation) [1-3].

Purified haemoglobin solutions

The first step in the pursuit for red cell substitute was...
to produce Hb in a mass scale and under sterile condition efficiently, ensuring high purity but no denaturation. The typical methods used were crystallisation of stroma free haemoglobin (SFH) obtained by hypotonic haemolysis; high performance liquid chromatography (HPLC); utilising the high stability of carboxylhaemoglobin against heating and dialysis (Somatogen Co) produced by *Escherichia coli* [4]; and transgenic human Hb produced by transgenic swine (Swanson et al; DNX Co) [5].

**Modified haemoglobin**

**Polymerised Haemoglobin**:

Initially, glutaraldehyde (a polymerising reagent for proteins) was used to produce PLP-crosslinked polymerised Hb with an adequate oxygen affinity [6]. Since the number of molecules remained less, the colloidal osmotic pressure could be kept low (20 Torr, \([\text{Hb}] = 15 \text{gm/dl}\)), thus enabling its transfusion as a solution with high Hb concentration. It had a circulation half-life of 24 hours and could be stored for more than 1 year. The drawbacks were related to the distribution of the molecular weight, uncertainty of the position of reacted sites, and high viscosity of the solution.

Transient renal tubular vacuolation after multiple injections have also been reported. These problems were solved by using chromatographic separation techniques. Initial trials were conducted on adult healthy males in the United States, Guatemala and in Zaire [7]. In 1992, the phase I clinical testing of a modified solution was carried out by Northfield Laboratories after approval of the FDA. The maximum dosage tried was 0.6 gm/Kg (63 gm maximum). No abnormality such as vasoconstriction was noticed. In the phase I trial using polymerised bovine Hb, dosing of up to 0.2 gm/kg caused a rise in blood pressure and a decline in heart rate, resulting in suspension of the trial [1].

Currently, soluble, cell-free, o-rafininose cross-linked and oligomerised human Hb (O-r-poly-Hb)(Hemolink, Hemosol, Canada)(molecular weight ranging from 32-500 KDa) is in a phase III clinical trial for peri-operative use in cardiac and orthopaedic surgery (at doses from 25g (250 ml)/100 g (1000 ml) [2,8]. Its affinity for oxygen appears lower than normal blood and an n (Hill coefficient) value of about 1 indicates a very low degree of co-operativity. Probably related to the low \(O_2\) affinity value and to the high molecular weight, O-r-poly-Hb has been shown to induce lesser haemodynamic perturbations than other first generation modified Hbs.

**Polymer Conjugated Haemoglobin**:

This was produced by increasing the molecular weight of Hb, by bonding it with a water-soluble polymer defined and distribution of the number of conjugated POE chains. Since this is a polymer-conjugated type, viscosity is high and the colloidal osmotic pressure is also high as 24 Torr, even at a Hb concentration of 8 gm/dl. The system that bonds bovine Hb may result in problems of antigenicity on repeated usage [9]. Other conjugated Hbs like, dextran-benzene-tetracarboxylate-conjugated Hb (Hb-Dex-BTC), is undergoing animal trials [10].

**Intramolecular Cross-Linked Haemoglobin**:

Diaspirin (3,5-dibromosalicyl fumarate; DBBF) was used to cross-link, \(\alpha\)-units and, \(\beta\)-units of Hb, and form diaspirin cross-linked Hb (DCL Hb). It was thought that cross-linking would prevent its dissociation and increase half-life during circulation (mean half-life is 10 hours). The viscosity of the solution is very low (2 cP), and the colloid osmotic pressure 28 Torr. At present, a production facility for mass yield is in operation. Phase I trial studies conducted by Baxter in 1992 have revealed considerable success, excepting a dose-related elevation of blood pressure and decrease in heart rate, corresponding to maximum dosage of 0.1 gm/Kg [1]. O’Hara et al observed an increase in whole blood methaemoglobin fraction (0.84 +/- 0.77% at baseline to 4.08 +/-1.36%) during 48 hours of the DCL Hb infusion (dosage :936 +/-276 mg/Kg), but it did not reach a range associated with complications [10-12].

**Recombinant Haemoglobin**:

Recombinant Hb (r Hb 1.1) is a method in which a few parts of an amino acid sequence of human Hb are replaced to prevent the dissociation into dimers and to maintain adequate oxygen affinity. The phase I clinical trial in 1992 recorded side effects like fever, chill and headache. By improving on the purification process, the endotoxin component was eliminated, thus reducing the adverse effects. Further trials in 1993, with a maximum dosage of 25.5 gm, recorded rise in blood pressure and mild gastrointestinal symptoms [1,4]. The phase II trials involved testing of a newer variant ie. rHb 414, where two rHb 1.1 were conjugated to obtain a double oxygen carrying capacity and a five times prolonged half-life per unit volume [1].

**Haemoglobin Vesicles**:

The advantages of Hb vesicles are: 1) no denaturation or modification of the Hb; 2) made of purified Hb and lipids. To simulate Hb encapsulation within RBCs, microcapsules made of nylon, collodion, gelatin, gum arabic and silicon were tried, but they were rapidly removed by the reticuloendothelial system. In 1977, Djordjevich and Miller [13] successfully used the theory of phospholipid vesicles for use in encapsulation and prepared Hb vesicles out of phospholipids, fatty acids,
chol sterol etc. [1]. Present research is focussed on liposome-encapsulated Hb (LEH) sterilisation, control of size, effective encapsulation and stabilisation of the vessels [12]. Recently a biodegradable polymer membrane-like polylactide has been used to develop Hb nanocapsules [8].

**Toxicity**

Cell-free Hbs, chemically altered or genetically expressed in microbial host systems (i.e. unmodified SFH), possibly cause excessive filtration of Hb dimers and tubular obstruction leading to a decreased glomerular filtration rate and renal damage. The major concern pertains to the interference of Hb and its oxidation products with the vascular redox balance, potentially impeding its clinical usefulness. Endotoxin contaminants, residual cellular stroma, and lipid fragments are known mediators of toxicity leading to vasoconstriction, complement activation and generation of free radicals [1,10,14,15].

**Perfluorocarbons**:

PFC are chemically inert compounds consisting of fluorine-substituted hydrocarbons. Unlike Hb-based substitutes, PFCs have the following advantages: 1) they do not react with oxygen or other gases; 2) increase the oxygen solubility in the plasma compartment; 3) the dissolved oxygen is not subject to the effects of temperature, pH, 2,3-DPG etc. (thus the oxygen dissociation curve is linear); and 4) facilitate effortless transfer of oxygen from the red cells to the tissue. Since they are insoluble in water, need for an emulsification prior to intravenous use was felt. Some workers advocated use of albumin and lecithin, whereas Geyer et al used Pluronic F-68, which is a mixture of short chain polymers [15].

**First generation perfluorocarbons**:

The first commercially available PFC was Fluosol-DA 20% (Green Cross, Osaka, Japan). This used Pluronic F-68, as an emulsifying agent, and was able to maintain a balance between the oxygen carrying capacity and tissue retention. It comprised two PFCs, perfluorodecalin (PFD) and perfluorotripropylamine (FTPA). PFD was the primary component and oxygen carrier, whereas FTPA was to provide the much needed stability. Each of the two components had different half-lives, with PFD being only 3 to 6 hours, due to its rapid clearance. FTPA on the other hand, persisted in the tissue for months [15].

**Second generation perfluorocarbons**:

Fluosol had paved the path for further refinement of PFCs. The desirable characteristics in the second generation PFC, as advocated by Reiss and Le Blanc et al [16] were: 1) large oxygen-dissolving capacity, 2) faster excretion and less tissue retention, 3) lack of significant side effects, 4) increasing purity, and 5) large scale production and availability. The three candidates chosen as per these criteria were PFD, perfluorooctyl bromide (PFOB) and bis (perfluorobutyl) ethylene. PFOB, known in its emulsion as Oxygent, was favoured for clinical trials because of its stability and high excretion rate.

Linear PFCs like PFOB dissolve oxygen better than cyclic ones like PFD. The oxygen solubility is inversely proportional to the molecular weight and directly proportional to the number of fluorine atoms. 90% weight/volume emulsions of PFOB have the capacity to contain four times the amount of oxygen as compared to a first generation PFC. PFCs being chemically inert, are not metabolised but are removed from the circulation, within 4-12 hours, by the reticuloendothelial system. They are stored in the liver and spleen and subsequently exhaled through the lung. Reducing the particle size or increasing the concentration leads to increased tissue deposition and subsequent tissue damage [15,17].

**Toxicity**

The major problem with the first-generation PFCs was due to complement activation. An in-vitro comparison of lecithin-based PFD emulsions and pluronic-based PFD emulsions, showed the latter to be virtually non-toxic to peripheral human leukocytes, except causing mild to moderate inhibition of endotoxin-induced cytokine production. Lecithin-based PFD emulsion caused substantial cytotoxicity in phagocytic cells like monocytes (60-100% after 24 hour incubation) and granulocytes (10-20% after 24 hour incubation). They also suppressed endotoxin-induced cytokine production in monocytes to more than 98% and inhibited cell proliferation of an endothelial (ECV 304) and a monocytic cell line (MonoMac6) to more than 95% [18]. However, an in-vivo trial of pluronic F-68 - based PFD led to adverse effects like intolerance of initial test dose, transient leucopenia, hypotension, chest pain, and a syndrome consisting of fever, leucocytosis and infiltrates on chest roentgenogram. In some cases there was an initial reaction to the test doses.

Acute toxicities due to the more refined second-generation PFCs, were in the form of transient facial flushing, backache, fever and a flu-like symptom within 1 to 4 hours post-infusion. These effects were attributed to the normal clearance of PFCs by the reticuloendothelial system. Opsonisation and phagocytosis of PFC droplets leads to activation of macrophages and release of prostaglandins and cytokines by the archidonic acid pathway [15,19]. Chronic or delayed side effects
were characterised by inhibition of platelet aggregation with 90% weight/volume PFOB emulsion, and transient thrombocytopenia (not below 80,000/µl). Owing to its organ retention effect, temporary histologic changes like enlargement and appearance of vacuolated histiocytes have been noted in liver biopsies [15,20].

**Potential clinical use**

Conventionally, it is recommended that, for haemorrhages less than 600 ml, plasma expanders should be used; for 600-1200 ml red blood cell products are necessary; and for blood loss above this, transfusion of plasma derivatives and/or platelet products, or whole blood is vital. Research with blood substitutes conducted on animal model and isolated human trials, recommends its usage to treat haemorrhages where blood loss is between 600-1200 ml. It should be used as an alternative and/or supplement to homologous and autologous transfusion, or in combination with the use of erythropoietin [15]. Various clinical, non-clinical and paradoxical utilisations have been envisaged (Table 1).

<table>
<thead>
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<th>Table 1 Potential clinical applications of oxygen carrying solutions</th>
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<td>1. Therapy</td>
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<td>(a) Blood substitutes: hemorrhagic shock; hemorrhage (war, surgery); anaemia.</td>
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<td>(b) Whole-body rinse out: acute drug intoxication; acute hepatic failure.</td>
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<td>(c) Local ischaemia: acute MI; evolving MI; cardiac failure; brain infarction; acute arterial thrombosis and embolism; PTCA of coronary artery.</td>
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<td>(d) General ischaemia: gas embolism; CO intoxication; HACP; HACO.</td>
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<td>(e) Aid for organ recovery: acute renal failure; acute hepatic failure; acute pancreatitis.</td>
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<td>(f) Infectious disease: anaerobic and aerobic diseases;</td>
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<td>(g) Adjuvant therapy: tumour radiotherapy; chemotherapy</td>
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<td>2. Perfusional protection of organs during surgery - cardiopulmonary bypass, deep hypothermia, circulatory arrest, cardioplegia.</td>
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<td>4. Drug carrier - drug-conjugated haemoglobin and perfluorochemicals.</td>
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<td>5. Contrast agent - (Perfluoro-octylbromide)</td>
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**Non-Clinical Applications**

1. Culture medium
2. Chemical examination - oxygen sensor; standard solution for oxygen calibrator
3. Bioreactor

**Paradoxical Utilisations (of high-oxygen affinity)**

1. Oxygen absorbent
2. Oxygen pulse therapy for malignant tumour in combination with radiotherapy or chemotherapy.

Clinical Use: The efficacy of Hb solutions in managing haemorrhagic shock and anaemia with low haematocrit, is better than crystalloid and colloid solutions. These have been used to preserve isolated organs, as cardioplegic agent, and as adjunct to preoperative autologous donation. A trial of bovine Hb solution in children with sickle cell anaemia, showed clinical improvement without any adverse effects. It has been tried in cases of organ recovery during organ failure, in infectious diseases, and as adjuvants in tumour therapies. PolyHb, DCLHb and recombinant Hb have been used in clinical trials of trauma patients [15,21,22].

Based on the effect of preserving aerobic metabolism during ischaemia and improving peripheral circulation by the oxygen carrying solution, these agents have been tried for perfusional protection of organs, Hb molecules and perfluorochemicals can be used to reach the blocked capillary beds, while increasing the drug sensitivity of the peripheral tissues by oxygenation. Perfluoro octylbromide can be used as a new contrast agent with oxygen carrying capability in ultrasound, CT scan, angiography, MRI, liver and spleen imaging and tumour imaging [8,22-25].

Non clinical applications: The oxygen carrying solution can be used in culture media of the tissues and bacteria (aerobes); in chemical examination such as an indicator for oxygen sensor, and a standard solution for oxygen calibrator; and in some kinds of bioreactor [25].

Paradoxical utilisations: If the oxygen affinity of the artificial red cells can be controlled, they can be used as an oxygen absorbent agent paradoxically. This can pave the way for a new form of tumour therapy i.e. “oxygen pulse therapy combined with radiotherapy and chemotherapy”. This would be in two stages. In the first stage, “low oxygen affinity solution”, will be used as an adjuvant therapy of radiation to deliver the oxygen to the tumours to increase radiosensitivity. The second stage would comprise “high oxygen affinity solution”, namely oxygen absorbent, delivered selectively to the feeding artery of the tumour expecting anoxic necrosis of the tumour [25].

**Present status**

Red cell substitutes are being developed for use in blood replacement therapies, either for perioperative haemodilution or for resuscitation from haemorrhagic blood loss [26]. There is an urgent need for these products because of risks associated with blood transfusions and pending worldwide blood shortages. Research in this field has benefited from development of new technologies in protein engineering. Clinical trials are being conducted to ascertain the safety and efficacy of these newly developed products [1,2,7,15]. Although cross-linked and polymerised products have been found to be safe in preclinical and early phase I/II clinical trials, they have had difficulty in proving efficacy. The primary adverse effect for the majority of cross-linked or polymerised products is a haemodynamic response, leading to increased vascular resistance to blood flow.
The physiological mechanisms are still incompletely understood, so that safety and efficacy cannot be completely dissociated. New understandings on the mode of action of these products will help to define their utility and application [27]. Only one product, a PFC compound, Fluosol (Green Cross, Osaka, Japan), has gained regulatory approval for limited use. Considerable progress has been made in developing platelet substitutes also. Lyophilised platelet, infusible platelet membranes, red cells bearing arginine-glycine-aspartic acid ligands, fibrinogen-coated albumin microcapsules and liposome-based agents have been developed as putative alternatives to the use of allogeneic donor platelet, to augment the function of existing platelets and/or provide a procoagulant material capable of achieving primary haemostasis in patients with thrombocytopenia [28].

Conclusion

The final goal of any transfusion service is to create a transfusion system with no side effects and with more effective medical care. The current system of homologous blood, although is marred by many problems, like allosensitisation and transfusion transmittable diseases, is working well with low cost, acceptable efficacy and relatively less side-effects.

Even so, the technologies of the future promise to generate an effective blood substitute, which will definitely have an impact on transfusion medicine and the transfusion services. Presently, the prospect of using artificial blood is difficult to envision owing to problems of short half life, prolonged tissue retention, potential toxicity, and issues of cost, cost relative benefits, availability of raw materials and strict FDA regulations. This may cause a temporary shift of focus from completely replacing blood cells, to that of using this as an adjunct to homologous transfusion. However, in prehospital or battlefield resuscitation, or in countries where supply of safe blood is affected by potential threat of HIV infection, the role of blood substitutes will undoubtedly be of immense value. And it is absolutely certain that a new system of blood service with artificial blood and blood substitutes, including artificial oxygen carriers and recombinant plasma components, will be developed in the near future, which will confer a new dimension to transfusion medicine.

References


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