Review Article

Alternatives to human blood transfusion-Reality or Dream?

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ABSTRACT

Blood transfusion is a cornerstone of medical practice especially in anemia and medical emergencies .Blood transfusion centers regularly face the challenge of donor blood shortage and risk of infectious transmissions of a variety of pathogens. History of blood transfusions have progressed through many developments. Researchers made significant effort to find an ideal alternative to blood transfusion with lot of work was focused on hemoglobin based oxygen carriers and perflurocarbons. Adverse effects restricted its use. With advances in technology utilization of recombinant factor like erythropoietin growth factor was developed. But it could not be fruitful for acute emergencies. Last 10 years, lot of research is being generated on utility of stem cells to produce red blood cells (ex vivo). Hematopoietic Stem cells have capacity of self-renewal and differentiation but it seems unlikely that these cells can provide sufficient volumes for large scale manufacture. However embryonic stem cells and induced pluripotent stem cells may fulfil this objective .Manufacture of red cells on large scale is promising but challenging proposal today. It could overcome the issues relating to sufficiency of supply, the risk of microbiological transmission especially the newer ones and issues related to immunological matching. Human trials with approval from FDA are scheduled in next couple of years, till then the health services are totally dependent on human donors.

Key words : Blood substitutes, perfluorocarbons, hemoglobin based oxygen carriers, stem cells.

Introduction -

Blood transfusion is a cornerstone of medical practice. There is always imbalance between demand and supply of blood especially in developing country where self-motivation for donation of blood is lacking. There are patients like thalassemia, aplastic anemia, hemophilia autoimmune hemolytic anemia where repeated blood transfusions are mandatory. To aggravate this situation Natural and man-made calamities need huge demand of blood units within short span of time. In addition there are situations like rare phenotypes of blood group, hemoglobinopathies making it difficult to fulfil the demand of blood. Although blood supply in many countries is safe, there is still certain risk of transmission of infectious agent during blood transfusion. As off today the donated blood is screened for Hepatitis B, C, HIV, malaria, filaria and syphilis. There is no way to test for prion transmitted diseases, or some other newer infections in the donated blood.

In view of all these considerations researchers are motivated to prepare ideal artificial blood or blood substitutes or synthetic blood. The criteria for an ideal blood substitute would be lack of antigenicity, ready availability, long half-life, storage at room temperature, excellent oxygen delivery to the tissues and very important affordable price to a common man. A lot of research is being generated to find an ideal blood substitute which goes way back in 1986 where Allen and colleagues used perflurocarbons in cases receiving radiotherapy to enhance oxygenation of the tissue[1]. Recombinant growth factors, hemoglobin based oxygen carrying substances (HBOC), Perfluorocarbons and platelet substitutes are the other alternatives experimented in past. For last decade, genetic engineering is exploring potential of stem cells, for ex vivo production of red blood cells a technique of "blood pharming".

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Agents that reduce perioperative blood loss.

The agents that reduce perioperative blood loss and hence necessity of blood transfusion include Desmopressin acetate vasopressin (1-deamino, 8D-arginine) DDAVP, topical agents like Fibrin gel /glue, agents that preserve platelet function and antifibrinolytic therapy. DDAVP is a synthetic analogue of antidiuretic hormone and increases plasma level of factor VIII and vWF. It is used in mild Hemophilia and Von Willibrand disease. A dose of 0.3mg/kg body weight increases factor VIII 3-5 fold[2]. Topical agents like Fibrin gel are directly applied to wounds or can be used to seal vascular grafts. Potential clinical applications include patients undergoing reoperative cardiac surgery, thoracic surgery and ontological surgery[2]. Infusion of antiplatelet agent dipyridamole has been reported to reduce platelet activation and depletion during cardiopulmonary bypass (CPB)[2]. Antifibrinolytic therapy includes € aminocaprionic acid, tranexamic acid and aprotinin and these have been tried to decrease blood loss and transfusion requirement in patients undergoing cardiac surgery[2].

Growth factors - Recombinant Human Erythropoietin (EPO)

Recombinant EPO has been shown to either eliminate or significantly reduce the RBC transfusion requirements of end stage renal disease and to correct hemostatic abnormality in them. EPO have additional use in HIV patients receiving zidovudine therapy, malignancy, and autoimmune diseases, prematurity and to facilitate the aggressive withdrawal of units of autologous blood over several weeks' time[1].

Current recommendations for target Hematocrit in a patient with CRF receiving EPO is 33 to 37% (Hb 11-12 gm/dl).Effects of therapy should be monitored every one to two week after starting EPO .Supplement iron to be given when % transferrin saturation is <20% and S.ferritin < 100ng/dl 1. Major limitations of EPO include anemia of acute onset and adverse effects like hypertension, headache, vomiting, aching in long bones.

Granulocyte / Monocyte Colony Stimulating Factor (GMCSF)

Recombinant GMCSF is a pan myeloid growth factor used following chemotherapy and marrow transplant, to accelerate myeloid recovery. It is also used to stimulate and increase CD34 cells in peripheral blood, of hematopoietic stem cells (HSC) donors. A dose of 600 microgm subcutaneously increases the yield of HSC four fold. It is given 12 hrs. before apheresis[1].

Thrombopoietic growth factor (TPO)

TPO is undergoing clinical development and has been found to improve the time to recovery of thrombocytopenia following nonmyeloablative chemotherapy and to increase yield of platelet of normal donors[1].

Hemoglobin based Oxygen carrying substitutes

Hemoglobin is the main component of red blood cells and hemoglobin based products are called hemoglobin based oxygen carriers. (HBOCs). Stroma-free hemoglobin has been investigated as an oxygen carrier since the 1940s, when researchers realized that native hemoglobin is not antigenic. A solution containing stroma-free hemoglobin has many advantages over red blood cells, including the ability to withstand sterilization and a shelf life of approximately 2 years at room temperature for some products. Solutions of acellular hemoglobin are not as effective at oxygenation as packed red blood cells because of their high affinity for oxygen. Unmodified free hemoglobin, when infused rapidly,

splits into dimers and is cleared by glomerular filtration and uptake by the reticuloendothelial system. Since half-life of stroma-free hemoglobin is quite short two methods are used to stabilize hemoglobin molecule. One uses intramolecular crosslinks to stabilize the tetramer. The other uses intermolecular crosslinks so as to produce a high molecular weight polymer of hemoglobin.

Diaspirin cross-linked hemoglobin (DCLHb) is the prototype molecule of this category of blood substitutes. DCLHb made from outdated human blood has a shelf life of approximately 9 months when frozen and 24 hours when refrigerated. The intravascular half-life is 2-12 hours and is dose dependent. Pyridoxylated hemoglobin is hemoglobin treated with pyridoxal-5-phosphate to reduce oxygen affinity and better release of oxygen to tissues. It has a half-life of 24 hours, a shelf life longer than 12 months when refrigerated. The first recombinant hemoglobin product, rHb 1.1 was a genetically engineered variant of human hemoglobin. The product was produced in *Escherichia coli* and had an intravascular half-life of 2-19 hours and a shelf life of 18 months when refrigerated. The adverse effect profile was similar to DCLHb[3].

The initial attempts at transfusing stroma-free hemoglobin produced nausea, vomiting, fever, general discomfort, renal dysfunction, coagulopathy, and hypertension. It is thought to result from hemoglobin binding to nitric oxide, which is a potent vascular endothelial relaxant[1]. Renal dysfunction is due to mechanical damage and associated vasoconstriction. Mechanical damage is due to glomerular filtration of hemoglobin monomers and dimers.

Efforts have been made to encapsulate hemoglobin within a lipid-membrane to create a compound capable of carrying oxygen while not being associated with significant vasoconstriction. These liposomes appear to be retained in plasma for a significant period. However, they are difficult to produce and can activate the reticuloendothelial system, the complement pathway, and platelets. Irrespective of several modifications in hemoglobin as blood substitute, adverse effects like hypertension, abdominal pain, skin rash, diarrhea, jaundice, hemoglobinuria, fever and stroke were observed. Though these effects were transient clinical trials were discontinued.

Perfluorocarbons -

Perfluorocarbons (PFC) are small size particles,1/70 the size of the diameter of RBC that act as intravascular oxygen carrier to temporarily augment oxygen delivery to tissues. This small size enable PFC particles traverse capillaries through which no RBC s are flowing. PFC can be used to deliver oxygen distal to partial vascular occlusion as in acute myocardial infarction, stroke and sickle cell crisis .PFC can also be used to increase oxygen content of the tumor in order to enhance subsequent treatment with ionizing radiation. Perfluorochemicals are chemically inert synthetic molecules that consist primarily of carbon and fluorine atoms, and are clear, colorless liquids.

The first generation PFC emulsion for administration to human was Fluosol-DA ®, a 20% w/v solution developed by the Green Cross Corporation (Osaka, Japan). Fluosol presented side effects, such as inhibition of white blood cells and complement activation, attributed to Pluronic[4]. Fluosol was used with success in around 300 patients who refused blood transfusion for religious reasons[5] and was authorized by the FDA for injection in humans (for percutaneous transluminal coronary angioplasty, PTCA). For several years Fluosol DA was permitted to be used in emergency situation. However in 1983 FDA questioned its use because of lack of convincing evidence that PFC were more efficient and concern over possible toxicity to RE system[1].

In the second generation emulsions, the PFC concentration is largely increased, enhancing so the O_2 - carrying capacity and eliminating the dilution of patient's blood at time of administration. These emulsions are formulated "ready-for-use" in buffered saline, and present a high stability[6]. The small size of their particles (mean diameter : 0.2 µm, about 1/35 of erythrocyte) allows them to easily

maintain perfusion of all the capillaries of the microcirculation during states of local vasoconstriction and ischemia, when erythrocytes no longer circulate[7]. The archetypal second generation emulsion is OxygentTM (Alliance Pharmaceutical Corp., San Diego, CA), 60% w/v PFC emulsion based on the use of a linear PFC, perflubron (perfluorooctyl bromide). It can be stored at refrigerated temperature for two years. The presence of the terminal bromine atom lends lipophilicity to perflubron and a more rapid excretion as vapors by the lungs, limiting its persistence in tissues. Its administration is not associated with hemodynamic effects and does not activate complement. But it could produce a dose-dependent and transient flu-like syndrome four to six hours after infusion, which results from the phagocytosis of the emulsion particles by macrophages.

Numerous studies were performed in humans, particularly with Oxygent TM, enrolling more than 500 subjects in phases I and II. An important clinical application of Oxygent TM is its administration during surgery with acute normovolemic hemodilution (ANH), to allow reductions of the patient's hematocrit below currently accepted thresholds while maintaining or improving tissue oxygenation. In a phase II study in orthopedic surgery with ANH, the use of Oxygent allows the reversal of trigger for transfusion.

Phase III studies with ANH in cardiopulmonary bypass surgery and in high-blood loss non cardiac surgery showed a greater avoidance of transfusion, but were stopped for more serious adverse events in the PFC group, results which remain debated[8]. A new phase II clinical trial in major surgery was planned in France for the end of 2006, but financial problems arrested the study.

In November 2007, phase II clinical trials in major surgery were announced in China, with the purpose to maintain hemodynamic stability and improve post-operative organ function. At the beginning of 2008, the manufacturing technology of Oxygent was transferred to Double-Crane Pharmaceuticals Co. in China, and a pilot production started. In Russia, another PFC emulsion, Perftoran, similar to Fluosol, but with improved emulsifier and low size particles 0.07 µm), is still in clinical trials with success in improving hemodynamics, and in reducing ischemic damage and allogeneic blood transfusion[9,10]. Out of Russia, Perftoran was used in valvuloplasty surgery with ANH[11].

The advantages of PFC emulsions as blood substitute are absence of incompatibility and risk of transmission of infectious diseases long duration of conservation, easy access, absence of metabolism and more particularly no reaction and no binding with O_2 allowing easy tissue unloading, viscosity and rheologic parameters similar to those of blood, permitting the particles to flow through swollen and/or blocked capillaries, where red blood cells might not pass. Disadvantages of PFC are it lacks affinity for oxygen, and hence the necessity therefore for patients to be in high O_2 environment with the possible development of O_2 toxicity to the lungs. Potential blockade of the RE system (PFC clearance) and subsequent diminished clearing of the pathogens. Side effects of PFC emulsions such as complement and phagocytic cells activation, principally due to the surfactant, are no longer seen with the second generation emulsions using newer surfactants.

Future uses of PFC emulsions -

Optimal use of PFC emulsions in the future may consist of a combination of acute normovolemic hemodilution (ANH) preoperatively with application of an artificial oxygen carrier such as a PFC emulsion during the operation, a procedure termed Augmented-ANHSM. Besides the use of PFC emulsions to reduce allogeneic blood transfusions in surgery, there are numerous other potential future indications based on their potential to augment tissue oxygenation[12]. Such future indications will likely include treatment and prevention of cerebral ischemia, stroke, and cardiopulmonary bypass related cerebral adverse events, spinal cord ischemia, myocardial ischemia, cardiac arrest, percutaneous coronary angioplasty and cardiopulmonary bypass, acute limb ischemia, emergency surgery and trauma as long as no

allogeneic blood is available and decompression sickness. Other applications include the use of PFC emulsions to augment tumor oxygenation to render them more sensitive to radiation and chemotherapy, prevention or treatment of sequelae of air embolism and finally to improve organ preservation for subsequent organ transplantation[12].

Hematopoietic Stem Cells for ex-vivo production of red blood cells -

With the advent of technology, hematopoiesis has been widely studied ever so and researchers are attempting production of red cells from the progenitor cell i.e. Hematopoietic Stem Cell (HSC)[13,14,15]. Red blood cells production from HSC begin with use of CD34+ marker. CD34+ cells are normally seen in cord blood and peripheral blood apart from bone marrow[13,16]. These stem cells have a basic property of

- **Self-renewal :** the ability to go through numerous cycles of cell division while maintaining the undifferentiated state.
- **Potency :** the capacity to differentiate into specialized cell types. In the strictest sense, this requires stem cells to be either totipotent or pluripotentto be able to give rise to any mature cell type.

Researchers have been utilizing these properties for ex-vivo production of red blood cells from HSC. A study performed by Giarratane et al in 2005 describe a large scale ex-vivo production of mature human red blood cells using HSC[13]. Moreover the blood cells produced possess same hemoglobin content and morphology as do the native red cells. This study also contend that red cells that are produced have a near normal life span this is in contrast to the current hemoglobin based blood substitutes which are found to be deficient. However difficulty in mass production, enucleation and high financial cost restricted its usefulness[16].

In 2011 a team of scientists injected 2ml of cultured blood into a consenting patient[17]. The injection was autologous, initial HSC were isolated from peripheral blood drawn from the patient. This was major breakthrough, demonstrating that RBCs produced in vitro survive at least as long as RBCs obtained by blood collection and behaved same way.

Following Giarratane study, several researchers are motivated for ex-vivo production of red cells using different techniques[18]. Basic steps for production of red blood cells from stem cells are

Step 1 - Isolation of CD34+HSC from cord blood or peripheral blood.

Step 2 - Culture of CD34+HSC with different cytokines, recombinant Erythropoietin, Stem cell factor, for proliferation and differentiation.

Step 3 - Culture with only EPO and poloxamer to produce red cell differentiation.

Step 4 - Enucleation of cells by 10th day.

Following Giarratane several industries and government bodies invested money for large scale manufacture of red cells[18]. In 2009 Defense Advanced Research Projects Agency (DARPA) in USA began 'blood pharming' program[19]. DARPA collaborated with Arteriocyte Inc. a biotech company[19]. This company was awarded nearly $2 million to manufacture the blood and to this effect company did the first shipment to FDA in 2011. They showed the ability to turn one unit of umbilical cord blood into 20units of blood in about 3 days at a cost of about $5000/unit. If FDA approves this "pharmed blood" donated blood is likely to be replaced in next five years. The projects are being undertaken in USA, England, Japan and Australia[17,20].

Red blood cells can also be generated from human embryonic stem cells (HESC)[21]. HESC are isolated from inner mass of blastocyst in a developing embryo. Embryonic stem cells can differentiate into all the specialized cellsectoderm, endoderm and mesoderm and maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues. However use of embryonic stem cells posed ethical problem in regards to procurement of these cells.

In 2010 induced pluripotent stem cells (iPSCs) were studied for therapeutic use[22,23]. Induced Pluripotent stem cells offer unique advantage to the researchers

to choose the origin of the cells. Peryard et al suggested that, 15 iPSC lines, representing most useful RBC phenotypes would be sufficient to manage 100% of all immunized patients[23] Induced pluripotent stem cell involves induction of pluripotency in tissue cells through transfection with combination of transcription factors. Initially murine fibroblasts were used, however researchers are now using induction in human fibroblast to produce red blood cells.

A team of scientists from the department of engineering design (IIT Chennai, India) has been successful in creating enough red blood cells from stem cells to be used as 'artificial blood' in people who need transfusion. Having proved their oxygen-carrying capacity, the RBCs will now go into 'mass production' before starting human trials in three years. The IIT team recently got a funding approval from the Union ministry of science and technology to produce artificial blood on an industrial scale[24]. This blood would be tested on animals before human trials. If the trials prove successful, this is going to be a reality dream of transfusion hematologists very soon.

Even though embryonic cells or induced pluripotent stem cells have unlimited capacity of self-renewal and differentiation, risk of infection and neoplasia could be high especially with iPSCs[20].

Till date there are not any approved human trials and the quantitatively engineered blood is underway in another 10-15 years[25].

Platelet substitutes -

Ideally platelet substitutes should be as effective as platelet transfusion in preventing and controlling bleeding in patients with quantitative or qualitative platelet defects and free of adverse effects. These include HLA stripped platelets which lose HLA antigen on treatment with chloroquine. The other substitutes include liquid cold stored platelets, lyophilized platelets, infusible platelet membranes, RBCs coated with fibrinogen, fibrinogen coated albumin microspheres, liposome based agents, factor Xa and phospholipid vesicles, or platelets produced in vitro. Due to the high utilization of platelets by patients undergoing chemotherapy or receiving stem cell transplants, platelet transfusion has steadily increased over the past decades. Human platelets can be obtained in vitro from the controlled differentiation of hematopoietic stem cells. However, the hemostatic quality of such manufactured platelets has not been confirmed and current technologies are inadequate to ensure satisfactory expansion and platelet biogenesis on an industrial scale[1].

Respirocytes -

Respirocytes are hypothetical, microscopic, artificial red blood cells that can emulate the function of its organic counterpart, only with 200 times the efficiency, so as to supplement or replace the function of much of the human body's normal respiratory system. Still entirely theoretical, respirocytes would measure 1 micrometer in diameter. In the original paper by Robert Freitas, titled, "A Mechanical Artificial Red Blood Cell : Exploratory Design in Medical Nanotechnology" (1998), it was proposed that respirocytes would mimic the action of the natural hemoglobin-filled red blood cells[26]. The proposed design of the spherical nanorobot is made up of 18 billion atoms arranged as a tiny pressure tank, which would be filled up with oxygen and carbon dioxide making one complete transfer point at the lungs, and the reverse transfer at the body's tissues.

Conclusion -

Several alternatives are available for allogeneic blood transfusion. It starts with Pharmacological interventions that reduce blood loss during surgery. Growth factors are available but have specific indications and limitations. Hemoglobin based Oxygen carrying substances have been tried since very long. Several modifications have been done in hemoglobin substitutes to achieve beneficial effect of autologous blood transfusion but the adverse effects restricted their use. PFC are more useful in all ischemic conditions like acute myocardial infarction, stroke, etc.

The goal to procure an ideal blood substitute which has universal compatibility, immediate availability,

freedom from disease transmission and long term storage has always eluded researchers. With advent of genetic engineering, ex vivo production of red blood cells from stem cells blood pharming is knocking at the doorstep of modern era. To add to this list science fiction respirocytes is a remote possibility. The ex vivo produced blood, is a promising dream for patients and hematologists has not been tried on humans till date. Human trials with red blood cells produced by blood pharming are scheduled in next couple of years with permission of officials. If or when the project comes to fruition, it may help to augment the regular blood supply, particularly in times of shortage when there is a sudden increase in demand. But this is a long-term project, and health services do not want people to stop donating blood. As of today health services are 100 per cent dependent on our blood donors and their generosity, and that is not going to change any time soon.

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