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Abstracts

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From artificial red blood cells, oxygen carriers, and oxygen therapeutics to artificial cells, nanomedicine, and beyond

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Abstract

The first experimental artificial red blood cells have all three major functions of red blood cells (rbc). However, the first practical one is a simple polyhemoglobin (PolyHb) that only has an oxygen-carrying function. This is now in routine clinical use in South Africa and Russia. An oxygen carrier with antioxidant functions, PolyHb-catalase-superoxide dismutase, can fulfill two of the three functions of rbc. Even more complete is one with all three functions of rbc in the form of PolyHb-catalase-superoxide dismutase-carbonic anhydrase. The most advanced ones are nanodimension artificial rbc with either PEG-lipid membrane or PEG-PLA polymer membrane. Extensions into oxygen therapeutics include a PolyHb-tyrosinase that suppresses the growth of melanoma in a mice model. Another is a PolyHb-fibrinogen that is an oxygen carrier with platelet-like function. Research has now extended well beyond the original research on artificial rbc into many areas of artificial cells. These include nanoparticles, nanotubules, lipid vesicles, liposomes, polymer-tethered lipid vesicles, polymersomes, microcapsules, bioencapsulation, nanocapules, macroencapsulation, synthetic cells, and others. These are being used in nanotechnology, nanomedicine, regenerative medicine, enzyme/gene therapy, cell/ stem cell therapy, biotechnology, drug delivery, hemoperfusion, nanosensers, and even by some groups in agriculture, industry, aquatic culture, nanocomputers, and nanorobotics.

Keywords: Blood substitutes, polyhemoglobin, oxygen carrier, artificial cells, carbon dioxide, oxygen radicals, Nanomedicine

Artificial red blood cells

The first artificial red blood cells fulfilled the following three major functions of red blood cells: (1) oxygen transport (Chang 1957); (2) carbon dioxide transport (Chang 1964); and (3) antioxidant functions (Chang and Poznanski 1968). However, serious interest in this area did not start until the HIV-contaminated-donor blood crisis. By then, there was no time to carry out the much-needed basic research.

Hemoglobin based oxygen carriers (HBOC)

The urgency led to the development of different hemoglobin based oxygen carriers (HBOC) that have only one of the functions of red blood cells. One of these is based on the basic research on glutaraldehyde crosslinked polyhemoglobin (Chang 1971). The group from Northfield has independently developed glutaraldehyde crosslinked human polyhemoglobin and carried out very extensive clinical trials (Moore et al. 2009). The group from Biopure has independently developed glutaraldehyde crosslinked bovine polyhemoglobin and has also carried out very extensive clinical trials (Jahr et al. 2008). South Africa has approved the routine clinical uses of polyhemoglobin for a number of years and Russia has recently approved the routine clinical uses of polyhemoglobin. Another HBOC in the form of conjugated hemoglobin can be formed by the basic principle of crosslinking hemoglobin to polymer (Chang 1964,1972). The one in development is a PEG conjugated hemglobin (Liiu and Xiu 2008, Winslow 2006).

HBOC only has one of the three major functions of red blood cells. However, the risk/benefit ratios of polyhemoglobin have already been shown in situations where rbc is not available (Moore et al. 2009, Jahr et al. 2008). Furthermore, polyhemoglobin can be sterilized to be free from HIV or other infective agents and it can also be stored at room temperature for more than one year. This is compared to donor red blood cells that require storage at 4ºC for up to only 42 days. Furthermore, there are questions of whether this length of storage of donor blood would lead to adverse effects (Roa et al. 2005).

Oxygen carriers with antioxidant functions

In the meantime, studies are being carried out towards oxygen carriers with antioxidant functions to fulfill two of the three functions of red blood cells (D'Agnillo and Chang 1998, Powanda and Chang 2002). This is important for conditions with potential for ischemia-reperfusion injury (Alayash

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et al. 2007). One approach is in the form of PolyHb-catalase-superoxide dismutase (D'Agnillo and Chang 1998) that is effective in preventing ischemia-reperfusion inury in a hemorrhagic shock-stroke rat model (Powanda and Chang 2002). Hsia's group has extended this using hemoglobin with synthetic antioxidant enzymes (Buehler et al 2004). There is also important recent research to resolve the effect of HBOC on nitric oxide in those conditions with endothelial dysfunction (Yu et al. 2010).

Blood substitute with enhanced antioxidant functions that can transport both oxygen and carbon dioxide

Sims et al. (2001) carried out studies in animal using tissue CO_2 microelectrodes. They show that tissue CO_2 is not reflected by blood PCO_2. Furthermore, tissue CO_2 increases with severity of hemorrhagic shock and is correlated with survival.

We have therefore carried out research on polyhemoglobin-catalase-supoxide dismutase-carbonic anhydrase (Bian et al. 2011). This fulfills all three major functions of red blood cells in acting as O_2 and CO_2 carrier with enhanced antioxidant properties.

Complete nanodimension artificial red blood cells

The original complete polymer membrane artificial red blood cells (Chang 1964, 1972) had very short circulation time. A study was carried out to prepare artificial cells with lipid membrane in the form of lipidprotein membrane and lipidpolymer membrane (Chang 1972). Djordjevich and Miller (1980) prepared submicron 0.2 micron-diameter artificial RBC using lipid membrane vesicles to encapsulate Hb. This increased the circulation time significantly. Philips' group (1992) markedly improve the circulation time by incorporating polyethylene-glycol (PEG) into the lipid membrane. The submicron hemoglobin lipid vesicle hemoglobin approach is being extensively developed by a group in Japan towards clinical trial (Tsuchida et al. 2006). Lipid vesicles would be useful for conditions that do not require a large volume of blood substitutes. Since the smaller the diameter the larger would be the surface-to-volume relationship, these 200 nanometer lipid vesicles have a total surface area and therefore lipid that is about 10 times that of seven micron red blood cells. A large amount of lipid can cause the saturation of the reticuloendothelial system. We therefore used biodegradable polymer membranes to form complete nanodimension artificial red blood cells (Chang et al. 2003). These nanoartificial RBCs of 80 to 150 nanometers contained all the red blood cell enzymes. Using a polyethylene-glycol-polylactide copolymer membrane, we were able to increase the circulation time of these nanoartificial red blood cells to double that of PolyHb (Chang et al. 2003). Further studies in rats by Liu and Chang (2008) show that one infusion with 1/3 the total blood volume did not result in any adverse effects or changes in the histology or blood biochemistries when followed on days 1, 7, and 21 after infusion.

Oxygen therapeutics

An oxygen carrier with enhanced antioxidants is not only for rbc replacement. It can be prepared with much higher antioxidant activities so that it can be used as oxygen therapeutic in conditions of severe ischemia-reperfusion, as in severe sustained hemorrhagic shock, myocardial infarction, stroke, or organ transplantation. An oxygen carrier with NO transport is another example of oxygen therapeutic, as is an oxygen carrier with platelet activity (Wong and Chang 2007). In high blood volume loss, replacement with a large volume of oxygen carrier or red blood cell alone would not replace platelets and coagulation factors. We have prepared a polyhemoglobin-fibrinogen that is effective as an oxygen carrier with platelet-like properties in rats with 98% volume exchange (Wong and Chang 2007). An oxygen carrier with anti-tumour activity in the form of PolyHb-tyrosinase has the combined function of increasing oxygen tension to sensitize the melanoma to therapy and lowering systemic tyrosine to retard the growth of this fatal skin cancer (Yu and Chang 2004).

Beyond oxygen carriers, blood substitutes, and oxygen therapeutics to artificial cells

The general principle of artificial cells can form the basis of a large number of artificial systems (Chang 2005, 2007) (Figure 1). In addition to being of cellular dimensions in the micron range, they can also be in the macro range, in the nano range, or in the molecular range. Furthermore, the membrane material includes polymer, biodegradable polymer, lipid, crosslinked protein, lipid-polymer complex, lipid-protein complex, and membrane with transport carriers. The artificial cells can contain an unlimited variety of material individually or in combinations (Figure 1). These include cells, stem cells, enzymes, multienzyme systems, hemoglobin, magnetic materials, microorganism, vaccines, genes for gene therapy, genetically engineered cells, adsorbents, drugs, hormones, peptides, proteins, and others. There have been increasing and explosive interest and research activities around the world on artificial cells, especially in fields related to biotechnology, nanomedicine, nanoscience, bioencapsulation, cell therapy, blood substitutes, advanced

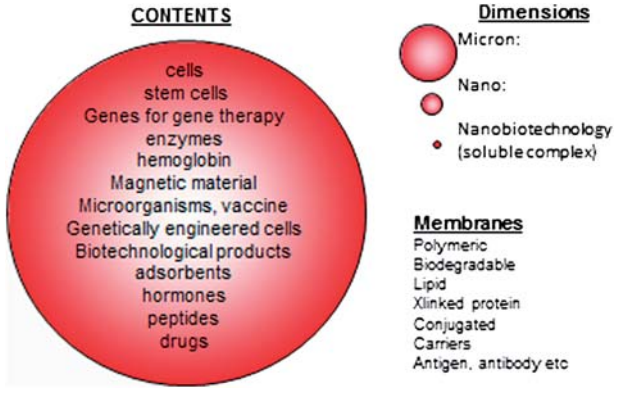

Figure 1. Possible variations in dimensions, membrane mateerials, and contents of artificial cells (from Chang 2007, with permission from World Science Publisher).

drug delivery systems, and even nanoscale robotics and others (Chang 2007). Some of these "artificial cells" are disguised under other terminologies such as liposomes, polymersomes, nanoparticles, microcapsules, blood substitutes, bioencapsulation, and so on.

Worldwide poll and artificial cells

In a recent worldwide poll by McGill University, the inventor of artificial cells was voted the Greatest McGillian in the university's 190-year history (www.artcell.mcgill.ca). This has less to do with the individual but is more related to the potential of artificial cells, including blood substitutes, that are being extensively developed by many groups around the world (Chang 2007, Liu and Xiu 2008, Mozzarelli 2010, Zapol 2012).

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Declaration of interest

The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

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July 27
Blood Storage Lesion — Nitric Oxide

Moderators: Christopher P. Stowell (MGH) and Walter H. Dzik (MGH)

CLINICAL STUDIES OF THE EFFECTS OF RED CELL STORAGE ON PATIENT OUTCOMES

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There are numerous studies consistently demonstrating biochemical, structural, inflammatory, and physiologic changes in refrigerated stored red cells, sometimes referred to as the "red cell storage lesion." It remains controversial, however, whether any of these changes have clinical relevance and actually impact clinical outcomes in transfused patients. More than 26 published clinical studies have evaluated the effects of red blood cell storage on patient outcomes. The most studied patient populations are cardiac surgery, critical care, and trauma. The majority of studies are non-randomized observational studies with methodologic shortcomings that limit interpretation. In such studies, attributing patient outcomes to transfusion is confounded by the underlying clinical condition necessitating transfusion. Patients with greater co-morbidity are more likely to be transfused and thus more likely to receive an older unit. Additionally, there is no scientific basis to define storage age in patients who receive multiple units stored for different periods. Lastly, studies frequently examine several patient outcomes and several definitions of "older blood" creating chance opportunities for an association with transfusion. There are three small randomized studies, none of which showed an adverse effect of red cell storage duration. Taken together, the body of data show mixed results with the majority of studies showing no adverse association of patient outcomes with longer storage and some showing a detrimental effect with longer storage. The published clinical studies evaluating the effects of red blood cell storage fall well short of providing sufficient evidence to change current clinical practice and reengineer the national blood bank inventory management system. These studies do, however, provide equipoise to support the ethical conduct of the large, definitive randomized clinical trials which are now underway.

CLINICAL CONSEQUENCES OF PROLONGED RED CELL STORAGE: ABLE STUDY

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(Abstract is not available)

ASSESSING THE CLINICAL EFFECT OF RBCS IN NEONATES: THE AGE OF RED BLOOD CELLS IN PREMATURE INFANTS (ARIPI) TRIAL

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Background: Despite recent trends in decreasing transfusion thresholds and the development of technologies designed to avoid allogeneic exposure, allogeneic red blood cell (RBC) transfusions remain an important supportive and life-saving measure for neonatal intensive care patients experiencing illness and anemia of prematurity. However, a number of laboratory and observational studies have indicated that the amount of time RBCs are stored has an impact on O_2 delivery to tissues. Consequently, older RBCs may result in higher rates of organ dysfunction, nosocomial infection, and lengths of stay. Because of potential harmful effects, an evaluation of the association between age of blood and nosocomial infection and organ dysfunction is warranted.
Methods: ARIPI is a double-blind, randomized controlled trial conducted at six tertiary care NICUs across Canada. The trial will be an effectiveness study evaluating the effectiveness of stored versus fresh RBCs in neonates requiring transfusion. Neonatal patients requiring at least one unit of RBCs will be randomized to receive either: 1) RBCs stored no longer than seven days; or 2) standard practice. All blood products will be supplied by the hospital blood bank. The primary outcome for this study will be a composite measure of major neonatal morbidities (necrotizing

enterocolitis, retinopathy of prematurity, bronchopulmonary dyspalsia, and intraventricular hemorrhage). Secondary outcomes will include individual items of the composite measure, nosocomial infection (bacteremia, septic shock, and pneumonia), and mortality.
Results: A total of 340 infants of an expected 370 have been enrolled to date. Aggregate baseline characteristics and outcomes will be presented.
Conclusion: As no randomized clinical trials have been conducted assessing the clinical effectiveness of fresh versus stored RBCs, the purported benefits of fresh blood remain unknown. The ARIPI trial aims to elucidate the clinical impact of stored versus fresh RBCs.

ADDRESSING THE QUESTION OF THE EFFECT OF RBC STORAGE ON CLINICAL OUTCOMES: THE RED CELL STORAGE DURATION STUDY (RECESS)

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Background: The question of whether storage of red blood cells (RBCs) alter their function when transfused into patients and affect clinical outcomes remains unanswered. The results of several largely retrospective patient studies have not been conclusive, with some finding an association between adverse clinical outcomes and the transfusion of RBC stored for longer periods of time, while others find no effect. The Red Cell Storage Duration Study (RECESS; NCT00991341), which is being conducted by the Transfusion Medicine and Hemostasis Clinical Trials Network supported by the NHLBI, is one of several multicenter, randomized, controlled trials designed to test the hypothesis that the duration of RBC storage affects clinical outcomes.
Materials and Methods: Patients undergoing complex cardiac surgical procedures are being randomized to receive RBC units stored for either $\leq$10 days or $\geq$21 days. Randomization may only occur if the blood bank has enough units of RBC of both storage times to meet the crossmatch request. The primary outcome is the change in the Multiple Organ Dysfunction Score (MODS), a composite measure of graded multiorgan dysfunction, by day 7. Secondary outcomes include the change in the MODS by day 28, all-cause mortality, and several composite and single measures of specific organ system function. The estimated total sample size required will be 1434 evaluable subjects (717 per arm).
Results: The first sites were activated on 6/21/10. Updated enrollment figures will be presented at the meeting.
Conclusions: Our understanding of the relationship between RBC storage time and clinical outcomes is in a state of equipoise. RECESS is among several clinical studies which are being conducted to provide the data for making evidence-based decisions about transfusion practice.

IMPACT OF TRANSFUSION OF AUTOLOGOUS 7- VERSUS 42-DAY-OLD AS-1 RED BLOOD CELLS ON TISSUE OXYGENATION AND THE MICROCIRCULATION IN HEALTHY VOLUNTEERS

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Background: Transfusion of stored red blood cell (RBCs) has been associated with increased morbidity and mortality when compared to transfusion of RBCs that have been stored for shorter periods of time. Stored RBCs accumulate biochemical and biophysical changes that may account for these adverse outcomes. The purpose of this study was to test the impact of transfusion of 7- versus 42-day-old stored, autologous RBCs on tissue oxygenation and the microcirculation in healthy subjects.
Materials and Methods: This was an observational, prospective study of healthy subjects who had two units of PRBCs collected in CP2D via venous apheresis. After collection the RBCs were leukoreduced and then stored at Duke University's Transfusion Services in AS-1 additive solution. Subjects were randomized to have transfused one of their two units of autologous RBCs, which had been stored for either 7 or 42 days following collection. Before, during, and after the transfusion, measurements of tissue oxygenation in the brain ($SctO_2$) and thenar eminence (StO_2) were recorded with non-invasive devices that use near infrared spectroscopy (NIRS). Additionally, sublingual microvascular blood flow was quantified prior to and after RBC transfusion using the Cytoscan® video microscope.
Results: To be presented at the meeting.
Conclusion: To be presented at the meeting.

NITRIC OXIDE TRANSPORT IN BLOOD: A THIRD GAS IN THE RESPIRATORY CYCLE

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S-nitrosylation, the covalent modification of a cysteine thiol by an NO group (to form an S-nitrosothiol or SNO), conveys a ubiquitous influence of nitric oxide (NO) in cellular signaling and physiology. Accumulating evidence indicates that pathophysiology is often associated with altered S-nitrosylation of specific protein targets (termed nitrosative stress). Hemoglobin provides a paradigmatic example of protein S-nitrosylation in which the allosterically regulated delivery of NO bioactivity subserves hypoxic vasodilation by red blood cells. Aberrant S-nitrosylation of hemoglobin is implicated in an array of human pathophysiologies characterized by altered red blood cell vasoactivity, and restoration of S-nitrosohemoglobin levels is central to the development of novel therapies that target the microcirculation and normalize oxygen utilization in the respiratory cycle.

NITRIC OXIDE AND THE RED CELL STORAGE LESION

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Background: Intravascular red cell hemolysis impairs nitric oxide (NO)-redox homeostasis, producing endothelial dysfunction, platelet activation, and vasculopathy. Red blood cell storage under standard conditions results in reduced integrity of the erythrocyte membrane, with formation of exocytic microvesicles or "microparticles" and hemolysis, which we hypothesized could impair vascular function and contribute to the putative "storage lesion" of banked blood.
Material and Methods: Packed red blood cell units, non-leukoreduced and preserved in ADSOL solution, were obtained from the Central Blood Bank, Pittsburgh, PA. Heme concentrations were determined spectrophotometrically and NO scavenging was determined by chemiluminescence. Stopped-flow spectroscopy and laser-triggered NO release from a caged NO compound were used to study reaction of NO with free hemoglobin and microparticles.
Results: We find that storage of human red blood cells under standard blood banking conditions results in the accumulation of cell-free and microparticle-encapsulated hemoglobin which, despite 39 days of storage, remains in the reduced ferrous oxyhemoglobin redox state and stoichiometrically reacts with and scavenges the vasodilator NO. Both free hemoglobin and microparticles react with NO about 1000 times faster than with intact erythrocytes. In complementary *in vivo* studies we show that hemoglobin, even at concentrations below 10 μM (in heme), produces potent vasoconstriction when infused into the rat circulation, while controlled infusions of methemoglobin and cyanomethemoglobin, which do not consume NO, have substantially reduced vasoconstrictor effects. Infusion of the plasma from stored human red cell units into the rat circulation produces significant vasoconstriction related to the magnitude of storage related hemolysis.
Conclusions: The results of these studies suggest new mechanisms for endothelial injury and impaired vascular function associated with the most fundamental of storage lesions, hemolysis.

TRANSFUSION OF STORED AUTOLOGOUS BLOOD ALTERS REACTIVE HYPEREMIA AND CIRCULATING NITRITE LEVELS IN HEALTHY VOLUNTEERS

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Background: Transfusion of blood stored for over two weeks is associated with increased mortality. During storage, red blood cells progressively release hemoglobin, which avidly binds nitric oxide. We hypothesized that the nitric oxide mediated hyperemic response following ischemia would be lower after transfusion of blood stored for longer periods of time.
Materials and Methods: We conducted a cross-over randomized interventional study, enrolling 10 healthy adults.

One could not donate blood a second time because of a low hemoglobin concentration and was therefore excluded. Each volunteer received one unit of 40-day and one of 3-day stored autologous leukoreduced packed red blood cells on different study days according to a randomization scheme. Blood withdrawals and reactive hyperemia measurements were performed before and 10 minutes, 1 hour, 2 hours, and 4 hours after transfusion.
Results: The reactive hyperemia index was lower (13% mean post-transfusion difference) after transfusion of 40-day as compared to 3-day stored blood ($p = .01$). Plasma cell-free hemoglobin and bilirubin levels were higher after transfusion of 40-day than after 3-day stored blood ($p = .02$ and .001, respectively). Plasma levels of potassium, LDH, haptoglobin, cytokines, and blood pressure did not differ between the two treatments and remained within the normal range. Plasma nitrite concentrations increased after transfusion of 40-day stored blood, but not after transfusion of 3-day stored blood ($p = .01$).
Conclusions: Transfusion of autologous blood stored for 40 days is associated with increased hemolysis and altered nitric oxide bioavailability, possibly due to enhanced scavenging by cell-free oxyhemoglobin.

July 28
New HBOCs/Approaches— Novel Protective Strategies

Moderators: Robert Langer (MIT) and Michael Marletta (UC Berkeley)

NEW MATERIALS AND TISSUE ENGINEERING

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Approaches involving the synthesis and application of bioerodible polymers to serve as implantable scaffolds for mammalian cells to create new tissues and organs are being studied. This talk will discuss the design of new materials in particular – synthetic polymers with specific ligands attached to them, shape memory degradable polymers, and materials with reversibly switching surfaces – that may have applications in these areas. We will also examine the use of materials coupled with human embryonic stem cells or other cells, and the application of these approaches to the creation of new tissues. This approach has been used to create a variety of tissues such as liver, skin, nerves, blood vessels, cartilage, and other tissues in animals and humans.

H-NOX: A NITRIC-OXIDE NEUTRAL, TUNABLE OXYGEN DELIVERY TECHNOLOGY

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Background: H-NOX proteins are a recently discovered family of gas sensors. Oxygen-bound H-NOX proteins are chemically inert and surprisingly nonreactive with the natural vasodilator nitric oxide (NO). By contrast, hemoglobin-based carriers (HBOCs) are chemically reactive and rapidly scavenge NO contributing to systemic toxicities that have limited their therapeutic potential. H-NOX proteins represent a potential major breakthrough in overcoming safety concerns of prior oxygen delivery efforts, and could result in safe and effective treatments for diseases of oxygen deprivation.
Materials and Methods: To create a panel of H-NOX proteins with a range of oxygen-binding affinity, structure-guided mutations were made using standard techniques. Purified ferrous-oxy proteins were evaluated biochemically for oxygen-binding kinetics, nitric oxide reactivity, and auto-oxidation rates. Promising candidates were formulated in physiologically compatible buffers for *in vivo* studies in rodents to evaluate safety and efficacy using standard methods.
Results: Engineered H-NOX mutants exhibit oxygen affinities spanning 20 nM to greater than 50 M and nitric oxide reactivity $< 0.1\ s^{-1}$ compared to ~$700\ s^{-1}$ for hemoglobin. In normal Sprague-Dawley rats, euvolemic intravenous administration of a lead H-NOX candidate (1 g/kg) shows no increase in mean arterial pressure or alteration of cardiac output. At 48 hours post-infusion, there are no significant organ toxicities or clinical chemistry abnormalities. In models of tissue ischemia, the H-NOX candidate delivers substantial oxygen to eliminate signs of hypoxia.
Conclusions: The extremely slow NO reactivity of oxygen-bound H-NOX is unexpected and suggests that H-NOX will be significantly safer as a therapeutic than hemoglobin-based approaches. To develop clinical candidates, variants were tuned with the appropriate oxygen affinities for delivery to tissues. Indeed, preliminary *in vivo* safety and oxygen delivery data of lead candidates are highly promising. In summary, the H-NOX platform offers a revolutionary advance in developing safe and effective oxygen delivery therapeutics.

ENGINEERING RECOMBINANT HB-BASED OXYGEN TO BE MORE EFFECTIVE, SAFER, AND EASIER TO PRODUCE

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The key requirements for a Hb-based oxygen carrier (rHBOC) are: (1) moderate O_2 affinity and large dissociation rate constants for efficient transport in capillaries; (2) significantly reduced rates of NO scavenging to prevent hypertensive side-effects; (3) resistance to auto- and chemically induced oxidation to inhibit oxidative stress; (4) low rates of hemin dissociation to increase shelf-life and decrease *in vivo* toxicity; and (5) enhanced apoglobin stability for increased expression yields in bacteria. Our goals over the past 20 years have been to determine the underlying biochemical mechanisms behind these processes and then use these principles to engineer the α and β subunits to create a rHBOC with all five properties optimized. For the most part these goals have been achieved, particularly with respect to efficient O_2 delivery and low rates of NO dioxygenation. In our view, the key remaining problem is to enhance the production of holo-rHBOCs in *E. coli*. A rational mutagenesis approach has been used to enhance the stability of the globin itself; co-expression with efficient Gram-negative heme transporter genes has both increased yields and reduced degradation products; and further improvement may be possible when low levels of the alpha hemoglobin stabilizing protein are present during rHBOC induction. These strategies are leading to the design of specific third-generation, less-vasoactive rHBOC prototypes with increased resistance to oxidation and denaturation, enhanced expression yields, and hopefully reduced production costs.

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CROSS-LINKED BIS-TETRAMERS (OCTAMERS) OF HUMAN HEMOGLOBIN: ROUTES TO STATE-OF-THE-ART HBOCS

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Background: Chemical methods for stabilizing hemoglobin tetramers have largely been limited to highly specific reagents that produced cross-linked tetramers and non-specific reagents that give higher molecular assemblies in mixtures that also contain tetramers. Those species that were assessed in clinical trials appeared to have significant problems that may have resulted from species with molecular weights comparable to the hemoglobin tetramer. Chemical methods can efficiently produce cross-linked tetramers but higher assemblies are not readily produced.
Materials and Methods: New reagents for cross-linking and joining tetramers were produced. These were based on the cross-linked with isophthalyl esters at each tetramer joined by various types of connectors. An efficient route would use a bio-orthogonal approach based on Meldal's copper-catalyzed azide-alkyne cycloaddition reaction. These were obtained from a combination of hemoglobin with a reactive ester remaining that can be displaced by an amine-azide.
Results: A variety of coupling methods were successfully developed and the resulting coupled, cross-linked tetramers were assessed for oxygen affinity and cooperativity. Materials with excellent oxygenation cooperativity resulted with a range of oxygen affinity. Some of these were tested in animal models and were found to have very little effect on blood pressure, while having good oxygenation characteristics.
Conclusions: The production of cross-linked bis-tetramers is an efficient process. The materials have appropriate characteristics for clinically useful HBOCs based on current knowledge in the field.

CHARACTERISTICS OF HEMOGLOBIN VESICLES AS A CELLULAR-TYPE ARTIFICIAL OXYGEN CARRIER

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The most abundant protein in blood is hemoglobin (Hb, 12-15 g/dL in blood). This fact indicates that oxygen transport is the most crucial for life activity. Hb is compartmentalized in red blood cells (RBCs), and the intracellular Hb concentration is 35 g/dL. In spite of its abundance in blood, Hb becomes toxic once it is released from RBCs. Hemoglobin-vesicles (HbV) are artificial oxygen carriers that mimic the cellular structure of RBCs to replace the blood transfusion.

In contrast to other liposomal products containing antifungal or anticancer drugs, one injection of HbV in place of a blood transfusion is estimated as equivalent to a massive dose, such as several hundred milliliters or a few liters of normal blood contents. The fluid must therefore contain a sufficient amount of Hb, the binding site of oxygen, to carry oxygen-like blood. Encapsulation of Hb can shield various toxic effects of molecular Hbs. On the other hand, the liposomal structure, surface property for biocompatibility, the balance between the stability (during storage and circulation in blood) and instability (for

the prompt degradation in RES) must be considered to establish an optimal transfusion alternative (Sakai et al., Methods Enzymol. 2009; 465: 363–384).

HbV is much smaller than RBC (250 vs. 8000 nm), but it recreates the functions of RBCs: (i) The oxygen-unloading of HbV is slower than that of a cell-free Hb solution; (ii) The colloid osmotic pressure is zero. For a massive dosage, HbV has to be co-injected with or suspended in a plasma substitute such as albumin; (iii) The viscosity of HbV is adjustable to that of blood; (iv) HbV is finally captured by RES, and then degraded and excreted promptly; (v) Co-encapsulation of an allosteric effector regulates oxygen-affinity; (vi) Hemolysis is minimal during circulation and the lipid bilayer membrane prevents a direct contact of Hb and vasculature; (vii) Reaction of NO is retarded by an intracellular diffusion barrier, and HbV does not induce vasoconstriction.

The obvious advantages of HbV are that it is pathogen-free and blood-type-antigen-free; moreover, it can withstand long-term storage for stockpiling. HbV has a variety of potential applications, not only as a transfusion alternative but also as an oxygen or CO therapeutic fluid that cannot be attained by the present RBC transfusion. Last year we lost a great leader, Emeritus Prof. Eishun Tsuchida. But our efforts continue, aiming at eventual realization of HbV.

OXIDATIVE REACTIONS OF HEMOGLOBIN AND SOME ANTIOXIDATIVE PROTECTIVE MECHANISMS

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Site-specific modifications to free Hb have been observed under mild oxidative conditions and when the protein is challenged with hydrogen peroxide (H_2O_2) in vitro. Firstly, the heme is oxidatively modified, becoming covalently linked to the protein. Secondly, extensive globin chain cross-links and irreversible modifications of key amino acids have been observed in human Hb. These included oxidation of sulfur-containing amino acids including βMet55, βCys93 and βCys112, all being oxidized to the methionine sulfoxide and cysteic acid forms, respectively. Diverse and complex physiological pathways are normally deployed in the mammalian system to control Hb oxidative reactions when released from RBCs. Chief amongst them is haptoglobin (Hp), which chaperones Hb subunits to the macrophages for safe degradation. Recent research on the interactions between Hb and Hp under oxidative conditions revealed that Hp specifically shields these key amino acids on the Hb molecule, allows the heme to consume oxidants, and short-circuits the emerging and damaging radicals through a redox cycle of its iron. We have observed similar oxidative changes after infusion of cell-free and chemically modified Hbs and/or Hb co-administered with nitrite in exchange transfusion models. In recent follow-up animal studies, we showed that the infusion of Hb complexed with Hp prevents Hb-induced systemic hypertension and tissue oxidative injury. It may prove necessary to explore these protective clearing mechanisms to counter the toxicity associated with free Hb in hemolytic anemias and when used as oxygen therapeutics.

WHAT TO DO WHEN YOU KNOW THERE IS NO NO?

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Background: Acellular hemoglobin within the circulation can scavenge endothelium generated NO. As a consequence, the infusion of HBOCs and old RBCs that are prone to hemolysis can be expected to lower levels of vascular NO. Reducing levels of NO in the circulation promotes vasoconstriction, which in general is not a desirable outcome if the objective is enhanced tissue perfusion. The situation becomes more serious if there is a backdrop of diminished eNOS generated NO due to endothelial dysfunction from either transient or chronic inflammatory conditions. Under these conditions, NO scavenging of the already compromised stores of NO by acellular Hbs from either infused HBOCs or hemolysis of RBC's can become a major factor in promoting a poor transfusion outcome. The current presentation will address two strategies for compensating for depleted NO levels within the circulation: i) NO releasing nanoparticles; and ii) production of nitrite mediated bioactive NO from Hb's.

Sustained release of NO from nanoparticles. A platform for NO releasing nanoparticles has been developed. The particles utilize nitrite as the source of NO. NO is released only when the particles come in contact with water. The release profiles indicate slow sustained release of NO that is tunable by minor modifications of the platform. Systemic IV infusion of the particles has been studied in vivo through collaboration with Pedro Cabrales (UCSD). The studies show sustained: i) reduction of MAP; and ii) increase in NO levels in the breath with evidence of anti-inflammatory behavior within the circulation. The particles have been shown to reverse the vasoconstriction induced by infusion of polymerized bovine Hb and stabilize the circulation subsequent to induced hemorrhagic shock. The emerging pattern indicates that these particles can be used as an additive to transfused blood or HBOCs to minimize toxicity due to NO scavenging and inflammation.

Nitrite-mediated generation of bioactive NO from Hb. In vivo experiments by Cabrales and coworkers (unpublished) show that certain Hbs such as PEGylated Hb can reverse vasoconstriction due to NO depletion, through a nitrite-mediated pathway. In addition, met Hb appears

to be more effective than ferrous Hb in reversing the vasoconstriction. A detailed biophysical mechanism will be presented that describes how met Hb in the presence of both small thiol-containing molecules and nitrite can generate GSNO, which is the likely long-lasting mediator of the observed effects. The results indicate that HBOCs can be engineered to undergo an efficient reaction that can compensate for NO scavenging.

VASOACTIVITY AND INFLAMMATORY RESPONSE TO HBOC INFUSION IN MICE

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Background: To date, there is no safe and effective hemoglobin (Hb)-based oxygen carrier (HBOC) to substitute for red blood cell transfusion. Clinical application of HBOCs has been stymied by the adverse side-effects of nitric oxide (NO) scavenging. The precise mechanisms remain unclear. We hypothesized that reduced NO bioavailability due to endothelial dysfunction would enhance vasospasm after HBOC challenge.
Materials and Methods: PolyHeme® was provided by Northfield Laboratories (Evanston, IL), MP4-OX, and MP4-CO were provided by Sangart (San Diego, CA) and Hb-vesicles (HbV) were obtained from Dr. Hiromi Sakai (Waseda Bioscience Research Institute, Singapore). All Hb solutions were given as a top load (16% of blood volume) via a tail vein. Systolic blood pressure (SBP) was measured in awake standard-diet (SD) fed wild-type mice (SD-fed WT, C57BL6), WT mice fed a high-fat diet for 4 - 6 weeks (HFD-fed WT), and diabetic (db/db) mice by tail-cuff. Invasive hemodynamics measurements were obtained in anesthetized mice. Blood and tissue samples (lung and liver) were collected 2 h after HBOC infusion for analysis of plasma levels of interleukin-6 (IL-6), IL-10, or monocyte chemotactic protein-1 (MCP-1), and tissue levels of IL-6, tumor necrosis factor-α (TNF-α), tissue factor (TF) or heme oxygenase-1 (HO-1).
Results: In SD-fed WT mice, infusing MP4-OX or MP4-CO produced systemic hypertension and vasoconstriction, but infusion of PolyHeme or HbV did not. In HFD-fed WT mice or db/db mice (with endothelial dysfunction), infusing PolyHeme induced systemic hypertension, but there was none after infusing HbV. These findings were confirmed with invasive hemodynamic measurements obtained in anesthetized mice. Infusion of PolyHeme, MP4 or HbV at 2 h did not change liver and lung levels of mRNAs encoding IL-6, TNF-α, TF or HO-1. At 2 h, no changes were observed in plasma levels of IL-6, IL-10, or MCP-1 after PolyHeme or MP4 infusion.
Conclusions: Our results demonstrate that some HBOCs (e.g. MP4-OX or -CO) produce more hypertension (due to systemic vasoconstriction) than others (PolyHeme) in SD-fed WT mice. CO binding to MP4 did not prevent systemic vasoconstriction. However, in mice with endothelial dysfunction (db/db) minor NO scavenging effects become more obvious, and even PolyHeme produces vasoconstriction. HbV did not produce vasoconstriction in db/db mice. None of the agents produced elevated cytokine levels in tissues or plasma at 2 h. Future HBOCs should be studied in models with endothelial dysfunction.

BIOCHEMICAL PLATFORM FOR THE IDENTIFICATION OF ADVERSE EFFECTS TRIGGERED BY FREE HEMOGLOBIN IN GUINEA PIGS

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Background: Hemorrhagic events occurring in regions with poor health service or battlefields and the increasing blood demand in hospital practice undermine the future sustainability of blood supplies and call for the development of alternatives to transfusions. However, studies carried out over more than three decades have not yet led to safe hemoglobin-based oxygen carriers (HBOCs). Several adverse effects were evidenced by clinical investigations and scrutinized in a controversial meta-analysis. To better define the side-effects of existing and future HBOCs in preclinical experiments, we are setting up a robust safety platform, based on Guinea pigs as animal models. Biochemical analysis and organ proteomics are being carried out to understand the underlying mechanisms of HBOCs' toxicity.
Materials and Methods: Guinea pigs were untreated or underwent a 50% exchange transfusion with Gelofusine®, followed by seven days of blood testing. Hemochromocytometric analysis and biochemical assays were carried out for the determination of the levels of amylase, lactic dehydrogenase, creatinine, aspartic and alanine transaminases. Animals were then sacrificed and their organs (heart, liver, kidneys, and lungs) were explanted and stored at -80°C. Bottom-up organ proteomics was carried out.
Results: Biochemical assays were optimized to take into account the interference of hemoglobin present in the plasma samples that, in several cases, causes significant alteration of the actual values. The effect of the exchange transfusion model on the health state of the animals was primarily evaluated comparing untreated animals with animals treated with the plasma expander. A transient alteration of transaminases and lactic dehydrogenase was observed, with values returning to basal levels within seven days. Remarkably, over the observation period, an

increase of platelets was observed. The proteomics analysis on Guinea pig hearts based on 2D electrophoresis was optimized to extensively characterize the protein pattern and its changes after treatment.
Conclusions: Results provide the basis for the identification of proteins that signal molecular events triggered by free plasma hemoglobin or HBOCs, and transient alteration caused by a plasma expander. This platform can also be exploited for the investigation of hemolytic conditions due to either blood transfusion or pathologies, such as sickle cell anemia, thalassemia, autoimmune anemia and malaria.

July 29
Session 1: Pre- Clinical and Recent Clinical Studies of HBOCs

Moderators: Warren M. Zapol (MGH) and Kenneth D. Bloch (MGH)

INCREASING PLASMA VISCOSITY INSTEAD OF OXYGEN-CARRYING CAPACITY IN THE TREATMENT OF BLOOD LOSSES AND DEPLOYMENT OF "SUPRA PLASMA EXPANSION"

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Background: Blood substitutes are perceived as resuscitation fluids that provide intrinsic oxygen capacity to the circulation. Delivery of oxygen to the tissues, however, depends also on blood flow, the distribution of blood pressure, and the resulting functional capillary density (FCD), parameters not uniquely related to one another. This allows one to compensate decreased blood intrinsic oxygen carrying capacity while maintaining oxygen delivery by increasing tissue perfusion. This approach is shown to be experimentally realistic since the development of high viscosity "supra plasma expanders," formulated using dextran 500 kDa, hydroxyethyl starch 670 kDa, or alginates. They elevate plasma viscosity ~ 2 cp under low hematocrit conditions that result into lowering blood viscosity, although not to the extent resulting from using non-viscogenic plasma expanders. These fluids increase shear stress on the endothelium and production of nitric oxide (NO), causing dilatation, maintaining blood pressure through increased flow, increasing transmission of central pressure to the capillaries improving FCD. Polyethylene glycol conjugated albumin (PEG-Alb) is a new class of "supra plasma expander" with the same effects and lower viscogenic capacity.
Materials and Methods: Viscogenic materials were obtained from commercial sources. PEG-Alb was synthetized and produced at the Albert Einstein College of Medicine using proprietary extension arm conjugation technology. Experimental studies used the hamster window chamber model, allowing the analysis of systemic and microvascular phenomena in the awake subject.
Results and Conclusions: Both types of supra plasma expanders lower the transfusion trigger to the limit of oxygen supply limitation or 4 g/dl blood hemoglobin (Hct 11%). PEG-Alb is effective in conditions of moderate heart function impairment, while viscogenic plasma expanders require normal heart function. Supra plasma expanders as blood substitutes are inherently beneficial since they maximize tissue perfusion and promote an anti-inflammatory environment through increased NO production, avoidance of NO scavenging and oxygen free radical generation. Supra plasma expansion preserves microvascular function, rendering the organism less susceptible to the negative effects of blood transfusion on FCD. It is proposed that it is the critical intermediate step for significantly lowering the transfusion trigger and blood usage, and improving outcome in blood volume resuscitation.

REVIEW THE DESIGN PARAMETERS OF SANGUINATE (PEG-BHB-CO), PRECLINICAL APPLICATIONS AS A THERAPEUTIC HBOC

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This presentation will review the design parameters of SANGUINATE (PEG-bHb-CO), preclinical efficacy studies, toxicology studies, and current clinical status.

PRE-CLINICAL STUDIES USING *OXYVITA HEMOGLOBIN*: A PATHWAY TO CLINICAL APPLICATIONS AS A THERAPEUTIC HBOC

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The evolution of HBOC (hemoglobin-based-oxygen-carrier) development over the past several decades has seen essential design considerations incorporated into new molecular species with the intent of addressing crucial factors responsible for less than optimal results observed during earlier clinical applications of first- and second-generation HBOCs). One unique molecular design approach emerged from the laboratory of Professor Enrico Bucci and his team at the University of Maryland using a novel zero-linked polymerization technology. Specific chemical modification of this zero-linked approach by OXYVITA, Inc., scientists resulted in several vital physical, chemical, and physiological properties being incorporated into this "super-polymeric" hemoglobin molecule. Exhibiting a mean molecular weight of 17 MDa (light scattering) and a methemoglobin content of less than 3%, it delivers oxygen at a low P50 (6 mmHg) with no cooperativity (Hill coefficient, n = 1.0). A more extensive description of the physical and chemical properties of OxyVita Hb has recently been published.

The focus of this review is to present an overview of earlier pre-clinical studies involving the use of these zero-linked HBOCs (referred to as ZL-HbBv) as they have emerged since 2000. These studies, carried out by independent investigators at different institutions, employed the earlier preparations of zero-linked polymeric hemoglobins. Recently, several studies have been carried out using the OxyVita Hb modified for production by OXYVITA, Inc. This review will discuss how OxyVita Hb has addresed several physiological/clinical issues that have negatively impacted HBOC implementation, namely: a) acellular hemoglobin intravascular extravasation linked to NO binding; b) observations of increased MAP (mean arterial pressure); and c) evidence of adequate oxygen delivery by HBOCs.

During the past several years multiple independent *in-vivo* animal studies have been carried out with this zero-linked polymeric HBOC addressing the issues of interstitial extravasation and related increases in MAP, cerebral ischemia and blood flow, resuscitation, and coagulation behavior.

POLYNITROXYLATED PEGYLATED HEMOGLOBIN (PNPH): A NOVEL NEUROPROTECTIVE HEMOGLOBIN IN EXPERIMENTAL TRAUMATIC BRAIN INJURY RESUSCITATION

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Background: Novel approaches to resuscitation of patients with traumatic brain injury (TBI) plus hemorrhagic shock (HS) are needed. An agent that simultaneously limits fluid requirements, augments oxygen delivery, and mitigates secondary injury would be optimal. Hypothesis: Resuscitation with the multi-functional anti-oxidant, colloid, hemoglobin (HB) polynitroxylated pegylated hemoglobin (PNPH), of bovine origin, will reduce fluid requirements, improve acute systemic and cerebrovascular hemodynamics, and attenuate neuronal death after experimental TBI + HS in mice.
Materials and Methods: We carried out a battery of experiments using *in vitro* models in rat primary neuronal culture and multiple *in vivo* models of both TBI alone and TBI + HS in mice. We assessed extracerebral and cerebral physiological parameters after injury and resuscitation (MAP, blood gases, lactate, HB, brain tissue oxygen [$PbtO_2$], and intracranial pressure [ICP]. We assessed hippocampal neuronal death at 24h or 7d and brain tissue levels of PNPH at 24h via EPR spectroscopy.
Results: In culture PNPH was non-toxic up to 12μM, unlike native HBm which was neurotoxic. Surprisingly, PNPH was neuroprotective in *in vitro* models of glutamate/glycine toxicity and neuronal stretch. In isolated CCI in mice, PNPH normalized MAP after injury ($p < 0.05$ vs no treatment or saline), but did not attenuate neuronal death at 24h. In contrast, use of PNPH vs lactated Ringer's in resuscitation of TBI + HS in mice markedly reduced fluid requirements ($p < 0.05$), improved $PbtO_2$ ($p < 0.05$), and attenuated the progressive rise in ICP during resuscitation ($p < 0.05$). Addition of 100% oxygen augmented the benefit of PNPH. PNPH attenuated hippocampal neuronal death at 7d after injury in a model of TBI + volume-controlled HS (MAP ~40mmHg), but not in TBI + severe pressure-controlled HS (MAP = 25 - 27mmHg). Brain tissue levels of PNPH after CC in mice given 20mL/kg were 150 - 200nM; levels safely within the neuroprotective range based on our *in vitro* studies.
Conclusions: PNPH is a promising novel neuroprotecitve HB that represents a small volume resuscitation agent in experimental TBI + HS.

Session 2: Recent Clinical Studies of HBOCs

Moderators: Steve A. Gould (The Gould Consulting Group) and Walter H. Dzik (MGH)

POSTINJURY RESUSCITATION WITH HUMAN POLYMERIZED HEMOGLOBIN PROLONGS EARLY SURVIVAL: A *POST HOC* ANALYSIS

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Introduction: In the Phase III USA multicenter trial with human polymerized hemoglobin (PolyHeme®), Day 30 mortality was 13% in the PolyHeme group versus 10% in the Control ($p > 0.05$). However, clear analysis comparing PolyHeme alone to Crystalloid alone or to RBCs alone is obfuscated in the aggregate population by the majority of PolyHeme recipients who also received RBCs. To examine possible early benefit of PolyHeme, we examined time to death among non-survivors, and performed *post hoc* subgroup safety analyses.
Methods: Time to death was analyzed for the 86 non-survivors. The following subgroups of all 714 enrolled and treated patients were analyzed: (1) Patients receiving 1U PolyHeme ($n = 112$) without RBCs vs Controls ($n = 147$) receiving crystalloid without RBCs (POLYvCRYS); (2) Patients receiving up to 6U PolyHeme ($n = 152$) without RBCs vs Controls ($n = 218$) receiving up to 6U RBCs (POLYvRBC).
Results: Time to death was prolonged in the PolyHeme group during the first eight hours following injury. In the POLYvCRYS subgroup, Day 1 (2% v 5%) and Day 30 (3% v 5%) mortality were lower in the PolyHeme group. No patients had MI, and AEs/SAEs were comparable between groups. In the POLYvRBC subgroup, Day 1 (3% v 5%) and Day 30 (5%) mortality in PolyHeme were lower than/identical to Control, respectively. MIs were low and identical between groups (1%), and AEs/SAEs were comparable.
Conclusions: PolyHeme prolongs early survival, likely owing to early oxygen-carrying replacement. The safety of PolyHeme vs Crystalloid or RBCs alone was comparable. These subgroup analyses are useful when extrapolating the use of PolyHeme to settings where RBCs are needed, but not available.

CAN HEMOPURE® BE BOTH CARDIOTOXIC AND CARDIOPROTECTIVE? REVIEW OF EVIDENCE SUPPORTING THESE CONTRADICTORY ASSERTIONS

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Background: Hemopure® (HBOC-201) vasoactivity coupled with an imbalance of cardiac adverse events in a phase III orthopedic trial (*J Trauma*. 2008 Jun;64(6):1484–97) have contributed to the presumption that HBOC-201 and other hemoglobin based oxygen carrier (HBOC) formulations share an intrinsic cardiotoxicity responsible for increased risk of myocardial infarction (MI) and death in clinical trials. This interpretation, however, fails to consider the impact of clinical trial data that shows the existence of persistent anemia during HBOC-201 therapy. We test whether this theory of cardiotoxicity "holds water" and is able to explain all results including contradictory claims of myocardial protection.
Materials and Methods: We compared side by side the results of studies that support cardiotoxicity with results of studies that support cardioprotection and evaluated the differences.
Results: Claims of cardiotoxicity are largely based on interpretations of clinical trial safety signals that are assumed to be the result of vasoactivity (*Artif Organs*. 2009 Feb; 33(2):100–9), (*Clinics (Sao Paulo)*. 2009; 64(8):803–13), (*JAMA*. 2008 May 21; 299(19):2304–12). There is no data to support a link between HBOC-201-induced vasoactivity and cardiotoxicity. Although mild-to-moderate transient increases in blood pressure secondary to nitric oxide scavenging and vasoactivity occur, systemic blood distribution is modified following HBOC-201 administration to maintain blood flow to vital organs (*J Trauma*. 2009 Jul;67(1):51–60). Despite small sample sizes, preclinical studies (*Am J Physiol Heart Circ Physiol*. 2010 Mar; 2 98(3):H1103–13) and clinical trials (*EuroIntervention*. 2008 Mar; 3(5):600–9), (*EuroIntervention*. 2008 May; 4(1):99–107) unequivocally confirm cardioprotection.
Conclusions: Hypoxia-related cardiac adverse events resulting from under-treated anemia have a clinical presentation similar to cardiac events resulting from ischemia secondary to severe vasoconstriction and resultant low blood flow. Failure to recognize this has contributed to the theory that vasoactivity = cardiotoxicity. The litmus test for any theory is its ability to explain not only the results on which it is based but all other available data. The cardiotoxicity theory has failed this test completely. Similarly, the cardioprotection hypothesis alone cannot explain the imbalance of cardiac events in the phase III clinical trial. It's clear that other factors are at play and these should be taken into consideration to explain both negative and positive outcomes in HBOC-201 trials. The cardiotoxicity theory fails to fully explain cardiac adverse events in HBOC clinical trials, offers no clues as to the mechanism of such "toxicity," and in light of evidence of myocardial protection by HBOC-201 is a less plausible explanation of such adverse events.

Session 3: Oxygen Therapeutic Agents

Moderator: Warren M. Zapol (MGH)

OXYGEN THERAPEUTIC AGENTS: RESULTS AND UPDATE ON RECENT TRIALS

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Background: Most cell-free hemoglobin solutions developed as blood replacement fluids have failed to improve oxygen consumption due to premature oxygen release and vasoconstriction. MP4OX is a low-volume oxygen therapeutic agent (OTA) developed to improve perfusion and oxygenate ischemic tissues, reduce lactic acidosis, reduce organ dysfunction and failure, and improve outcome from hemorrhagic shock. MP4OX is formulated at a low Hb concentration (4.3 g/dL), with high oxygen affinity (P50 = 5 mmHg) and high colloid osmotic pressure (COP = 70 mmHg). Vasoconstriction has not been seen clinically, perhaps due to NO regeneration from the high nitrite reductase activity of MP4OX, which may be due to β-93 pegylation.

Published Phase 3 data from two studies have both demonstrated satisfactory safety and the excellent colloid property of MP4OX. These studies were not designed to study OTA properties given the low rate of oxygen debt incurred in appropriately managed primary hip replacement surgery patients.

Prolonged ischemia complicates traumatic hemorrhagic shock and is associated with worse outcomes in terms of mortality, organ dysfunction, sepsis, and hospital stay. We conducted a Phase 2a international multicenter randomized double-blind controlled trial of MP4OX, which investigated its safety and efficacy.

Materials and Methods: Patients in hemorrhagic shock from blunt and/or penetrating trauma with lactic acidosis (lactate ≥ 5 mmol/L) were enrolled within 2 hrs of arrival and 4 hrs of trauma at 11 centers in 4 countries (South Africa, UK, France, and Germany). Patients received either 500 mL Ringer's lactate (RL) or 250 mL MP4OX + 250mL RL or 500 mL MP4OX within 187 minutes of injury, in addition to standard care including all blood products.

Results: Groups were balanced for demographics and injury severity. Patients received 2.4 to 8.2 mL/kg MP4OX achieving plasma Hb levels of 8.8 g/L for MP4OX-250 (similar to 7.6 g/L in a preclinical swine aortic tear model; Young et al., *CCM* 2005;33:1794).

There were no significant differences in overall serious adverse events, cardiac events, or mortality. There were no differences in AST, ALT, amylase, lipase, troponin I, and creatinine levels. Baseline lactates were similar. More MP40X patients had an immediate lactate decrease seen by the end of infusion and this was sustained to Hour 2. MP4OX had a greater proportion of > 10% and > 20% lactate clearance between Baseline and Hour 2. There was no difference in lactate clearance between MP4OX-250 and MP4OX-500 groups. Trends toward more patients normalizing lactate by Hour 4, and having improved outcomes with reduction in median total hospital days and the proportion discharged from hospital by Day 28, were seen in MP4OX treatment groups.

Conclusions: MP4OX has a satisfactory safety profile in trauma and was associated with an immediate and sustained OTA effect on lactate clearance. There is sufficient evidence of trends in better outcomes to advance to pivotal studies in trauma.

Session 4: Stem Cell RBC Production

Moderator: David Scadden (MGH)

PROSPECT FOR STEM CELL BASED MEDICINE

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(Abstract is not available)

BIOENGINEERING FOR RED CELL PRODUCTION

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In the context of the constant difficulty of obtaining supplies of blood products, the interest of disposing of complementary sources of red blood cells for transfusion is evident. The development of chemical or natural molecules which would replace hemoglobin is proving difficult. Artificial blood is still unattainable. Hence instead of replacing what is made by nature, why not copy it? For these reasons, attempting to generate red blood cells in the laboratory makes sense. We'll describe the research in progress which will permit the large-scale production of human red blood cells from hematopoietic stem cells. We'll discuss the state of the art of this concept, evoke the obstacles to be overcome to pass from the laboratory model to clinical practice, and analyze the possible indications in the medium and long term. We'll discuss the potential

interest of pluripotent stem cells as an unlimited source of RBCs. If it succeeds, this new approach could mark a considerable advance in the field of transfusion.

PRODUCTION OF ERYTHRIOD CELLS FROM HUMAN ES AND IPS CELLS

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Human embryonic stem cells and induced pluripotent stem cells hold great promises for regenerative medicine because they can be grown in very large quantities and can theoretically be differentiated into all cell types and tissues. We will present our latest results on the differentiation of ES and iPS into hematopoietic stem and progenitor cells as well as into fully enucleated red blood cells. We found that it is possible to produce large amounts of enucleated red blood cells from both human ES and iPS cells. Detailed globin expression analysis revealed that ES cell differentiation in the erythroid lineage closely recapitulates normal development since we have developed conditions in which the ES cells differentiate into erythroid cells synthesizing successively predominantly Hb Gower I, HB Gower II and Hb F. The Hb F containing cells that we obtained are similar to cells that would be observed in a fetal liver at about 10 weeks of gestation. Although a small amount of beta-globin expression can be detected by PCR, It has not been possible so far to differentiate pluripotent cells into erythroid cells that would be similar to adult red blood cells that express over 99% adult hemoglobin (Hb A). The sequential expression of the embryonic and fetal hemoglobins was similar for ES and for iPS cells derived from embryonic or adult fibroblasts, suggesting that the age of the donor cells did not affect the phenotype of the iPS and that all iPS tested had been reprogrammed to an embryonic state and did not express any memory of having been adult upon differentiation into the erythroid lineage. We will also present our results on modification of iPS cells by zinc finger nucleases.

RED BLOOD CELL MANUFACTURE: CHALLENGES AND OPPORTUNITIES

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A ready supply of safe and effective red blood cells (RBC) is critical to the effective management of trauma and other disorders. Current RBC procurement practices that rely on the regular donation of whole blood are problematic due to the availability of donated blood units, relatively short shelf-life of donated units, potential time delays in transporting donated units point of use, and increasing possibility of transmitting pathogenic adventitious agents to the recipient. The ability to manufacture RBC outside the body from a screened stem cell supply could overcome many of the problems associated with whole blood donation. The current state of the art, challenges and future opportunities will be presented based on the experience and lessons learned from the initial stages of developing a large-scale continuous clinical production system for RBC.

POSTERS

Poster is selected for Young Investigator Award Oral Presentation

P-01

ENHANCING THE NITRITE REDUCTASE ACTIVITY OF MODIFIED HEMOGLOBIN: BIS-TETRAMERS AND THEIR PEGYLATED DERIVATIVES

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Background: One of the most vexing problems in the development of a safe and functional hemoglobin-based oxygen carrier (HBOC) is systemic vasoconstriction. This HBOC-induced hypertension is now attributed to nitric oxide (NO) scavenging. Recent reports indicate that the nitrite reductase activity of hemoglobin can serve as a major endogenous source of NO. Here we investigate the effects of protein modification on the rate of nitrite reduction. In this manner, the specific chemical alterations that enhance NO production via nitrite reduction as catalyzed by hemoglobin can be determined. Protein modifications that enhance the nitrite reductase activity of hemoglobin can serve to counteract consumption of endogenous NO by hemoglobin.
Materials and Methods: Cross-linked hemoglobin (e.g. DCLHb) and polyethylene glycol (PEG) conjugated hemoglobins were prepared by chemical methods. Bis-tetramers of hemoglobin and their PEG derivatives were produced by chemical acylation. Kinetic measurements of nitrite-heme reaction rates were used to assess the overall effects of chemical modification on the nitrite reductase activity of hemoglobin. A method for the deconvolution of spectral kinetic data by multi-linear regression analysis was developed in order to obtain rate constants in the kinetic study.
Results: Cross-linked hemoglobin (e.g. DCLHb) has heme-nitrite reaction rates similar to native hemoglobin. In contrast, addition of PEG chains at β-Cys93 results in an enhancement in the rate of nitrite reduction to NO. Native hemoglobin conjugated with multiple PEG chains at β-Cys93 further increases the rate of NO production. Conjugation of four PEG chains to bis-tetramers of hemoglobin produces a material with greatly increased nitrite reductase activity while retaining oxygen cooperativity.
Conclusions: PEG conjugated hemoglobins convert nitrite to NO at a faster rate than does the native protein, an effect that may compensate for the scavenging of NO. The enhanced production of NO comes from an increased proportion of the protein residing in the R-state. Our results show that PEG conjugated bis-tetramers combine increased size with enhanced nitrite reductase activity expected for decreased vasoactivity, characteristics that are important for an acceptable HBOC.

P-02

FLUID RESUSCITATION USING LARGE VOLUME OF HEMOGLOBIN VESICLE IN RAT CONTINUOUS HEMORRHAGE MODEL (2ND REPORT)

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Background: Hemoglobin vesicle (Hb-vesicle) is a cellular-type oxygen carrier and it is capable of transporting oxygen as equal as a red blood cell. In ISBS 2009, we have presented a study of a rat uncontrolled hemorrhagic shock model which has been resuscitated with Hb-vesicle. Rats survived during the fluid resuscitation for 2hr at speed of 56mL/kg/hr. However, that model needs a large volume of resuscitation fluid to maintain the blood pressure. We try to suppress the volume of resuscitation fluid and to maintain the functional hemoglobin concentration by addition of Hb-vesicle.
Materials and Methods: Hb-vesicle/saline suspension (HbV) and washed red blood cell/saline suspension (wRBC) was prepared and used as resuscitation fluid. Hb-vesicle was provided by Nipro Crop. (Kyoto, Japan). wRBC was made from homologous blood. Rats (n = 12) were anesthetized with 1.0 - 2.0% sevoflurane. To induce hemorrhagic shock, rats were continuously bled from caudal artery. After shock was established, fluid resuscitation had begun with HES and oxygen carrier (either wRBC or HbV). For suppression of usage of oxygen carrier, HES was infused through a femoral vein at a speed of 14mL/kg/hr, and oxygen carrier (either wRBC or HbV) was simultaneously infused through the caudal vein at a speed of 14mL/kg/hr. Volume of resuscitation fluid was determined to be 200% of estimated circulating blood volume. After resuscitation was finished, hemostasis was achieved and the difference of hemorrhage and resuscitation fluid was infused additionally with the oxygen carrier. Subsequently, maintenance infusion was administered. For the wRBC group HES/saline (5ml/kg/hr) and for the HbV group HES/HbV (5ml/kg/hr) was infused until the end of the experiment. Maintenance infusion was kept until 24hr after the resuscitation start. Measurement and blood collection were carried out at several points until the injection was stopped.
Results: In survival rate, the HbV group was 83.3% and the wRBC group was 100%. MAP at the end of

resuscitation was 45.7 ± 9.7mmHg in the wRBC group and 53.5 ± 6.6mmHg in the HbV group. After 24 hours' observation, MAP recovered to 84.0 ± 3.5mmHg (wRBC group) and 81.2 ± 12.7mmHg (HbV group). Cutaneous blood flow was significantly higher in the HbV group than the wRBC group after 120% BV (blood volume) was infused. Administered volume of HbV was 42.0mL/kg (resuscitation phase), 10.4 ± 5.2mL/kg (difference of hemorrhage and resuscitation), and 50mL/kg (maintenance phase).

Conclusions: We successfully suppressed HbV usage and prolonged the survival in the continuous hemorrhage model. Effective resuscitation was achieved using parallel infusion of HbV and HES with permissive hypotension.

P-03

NONCONSERVATIVE EAF-PEGYLATION OF ALBUMIN: CONJUGATION CHEMISTRY INFLUENCES STRUCTURE OF PROTEIN CORE AND HAS NO INFLUENCE ON PEG-SHELL PACKING DENSITY

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Background: PEGylation induced Plasma Expander (PE)-like properties of Albumin (Alb) and Hb make these a new class of PEs that achieves "active plasma expansion." PE-like properties are an inverse correlate of the packing density of PEG in the PEG-shell. PEG Alb and Hb generated by the conservative Extension Arm Facilitated (EAF) PEGylation platform that introduces the conservative amidine linkages between the protein amino groups and Extension Arms using 2-iminothiolane (IT) have been studied thus far. New nonconservative (NC) EAF PEGylation protocols that replace the amidine linkage between the protein and extension arms by an isopeptide linkage using active ester chemistry have been developed and the influence of the length of the EA and the chemistry of the linkage on PE-like properties of PEG Alb adducts have been delineated.

Materials and Methods: NC EAF PEGylation of Alb has been carried out using N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and Sulfo-LC-succinimidyl 6-(3'-[2-pyriyldithio]-propionamido) hexanoate (Sulfo LC-SPDP) as the heterobifunctional reagents to introduce EAs through isopeptide linkages with thiols at the distal. The lengths of EAs in these two cases are 6.4 Â and 15.7 Â, respectively, compared to 8.0 Â of 2-IT. [N-e-Maleimidocaproyloxy] sulfo succinimide ester (EMCS) has been used to introduce EAs with maleimide at the distal end (length 9.4 Â) to carry out EAF PEGylation using thio-PEG 5K. EAF hexaPEGylated Alb has been generated using the NC-strategies and the influence of the length and charge manipulations on PE-like properties and structure of protein core has been investigated.

Results and conclusions: EA chemistry has little influence on the packing density and the overall efficiency of the PEGylation induced PE-like properties. The new NC-chemistry has a noticeable influence on the conformation of Alb. Thus the aspect of the structure of the PEG shell that makes PEG-Alb a superior plasma expander is the function of pattern of PEGylation rather than the chemistry of EA conjugation. Accordingly, EAF PEGylation can be manipulated without much influence on the function of the "active supra perfusion" agents. The new flexibility designed here for EAF PEGylation presents an opportunity to modulate the functional properties of therapeutic proteins (O_2 affinity and/or cooperativity in the case of Hb). It is speculated that the influence of EA chemistry on the protein core is a consequence of the perturbation of the protein hydration layer influenced by the charge perturbation at the site of attachment. The new flexibility incorporated into EAF PEGylation should facilitate the reengineering of the hydration layer of the proteins to increase therapeutic efficacy.

P-04

NONCONSERVATIVE EXTENSION ARM INDUCES A BETTER THERMAL STABILITY TO EAF PEG-HB THAN THE CONSERVATIVE ONE

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Background: Developing vasoinactive Hb by engineering plasma expander-like properties to the protein by Extension Arm Facilitated (EAF) hexaPEGylation has emerged as a simple and cost-effective strategy. EAs engineered between PEG and Hb, in particular, attenuate the influence of PEG-shell on the quaternary structure of Hb, thereby minimizing the PEGylation-induced dissociation of PEGylated tetramers into dimers. δ-mercapto butyrimidate is the EA arm introduced onto the protein by 2-iminothiolane. The thiol is the targeted functional group for PEGylation with maleimide PEG. The EA conserves the positive charge of the ε-amino groups of thiolated Hb, the intermediate in EAF PEGylation. In an attempt to optimize EAF PEGylation platform for hexaPEGylation of Hb,

nonconservative versions of EAF PEGylation of Hb have been now developed and the influence of the conservative and nonconservative EAs in hexaPEGylated Hb on the thermal stability of Hb has been compared to that of direct nonconservative PEGylation of Hb.

Materials and Methods: EAGF PEGylation of Hb as described earlier using 2-iminothiolane (EA length ~8 Å) and maleimido phenyl PEG-5K has been used to introduce conservative extension arms. Reversible protection of Cys-93(β)of Hb as mixed disulfides of thiopyridine has been adopted to keep thiol Cys-free. Conservative EAF PEGylation controls have also been generated using N-ethylmaleimide (NEM) instead of maleimido phenyl PEG-5K. Nonconservative (NC) EAF PEGylation has been achieved using [N-e-Maleimidocaproyloxy] sulfo succinimide ester (EMCS) to introduce EAs with maleimide at the distal end of the EA (length 9.4 Å) targeted for PEGylation with thio-PEG 5K. Direct hexaPEGylation of Hb has been carried out using succinimido propionyl PEG-5K. Thermal transition patterns (TTP) of Hb and hexaPEGylated Hbs are generated using a Differential Scanning Microcalorimeter.

Results and Conclusions: Two transitions, Tm 1 and Tm 2, around 60°C and 70°C are seen in the TTP for Hb. These two transitions correspond to the perturbations of inter ($\alpha 1\beta 2$) and intra ($\alpha 1\beta 1$) dimeric interactions, respectively. Direct hexaPEGylation induces no significant changes in Tm1 and Tm2 of Hb. Conservative EAs in PEG-Hb either with or without reversible protection of Cys-93(β) lowers both Tms. EAF NEM control of Hb without protection of Cys-93(β) establishes that EA chemistry essentially contributes to the lowering of Tm. PEG chains play little role in this change in Tms. NC-EA arms broadens the transition, increases Tm1 to 69°C and Tm2 to 79°C. It is concluded that NC EAs stabilize inter dimeric interactions better, and are preferred in the EAF PEGylation platforms for generation of vasoinactive hexaPEGylated Hbs.

P-05

MACROMOLECULAR ANTIOXIDANT THERAPEUTICS WITH PEGYLATION-INDUCED SUPRA PERFUSION: STRUCTURAL AND FUNCTIONAL ADVANTAGES OF EXTENSION ARM CHEMISTRY FOR CONJUGATION OF ANTIOXIDANTS TO PEGYLATED PROTEINS

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Background: HexaPEGylated Hb and hexaPEGylated albumin represent a new class of low-viscosity supra perfusion plasma expanders that have documented maintenance of cardiovascular and microvascular function at high levels. Transfusion is generally associated with some degree of perturbation of the normal oxidative stress as seen in reperfusion injury. In addition, in many disease situations such as sickle cell disease, the pathophysiology of the disease mimics that of reperfusion injury. We have now translated our extension arm chemistry as a simple chemical approach to conjugate desired levels of antioxidants to these supra plasma expanders with minimal perturbation of the core protein structure and have developed novel macromolecular antioxidant therapeutics with excellent perfusion.

Materials and Methods: HexaPEGylated-Alb and hexaPEGylated Hbs are produced at the Albert Einstein College of Medicine using proprietary extension arm conjugation technology. Conjugation of antioxidants to albumin and Hb [thiol of Cys-93(β) reversibly protected as mixed disulfide with thiopyridine and release after PEGylation and/or polynitroxylation] and their hexaPEGylated derivatives were made in phosphate buffered saline (pH 7.4), at 4°C for six hours using 2-imnothiolane in the presence of maleimide derivative of the desired antioxidants. EAF P5K6 Albumin with 12 tempols and EAF-P5K6 Hb with six tempols were developed using this new approach. Extent of EA mediated polynitroxylation has been established by thiol estimations in control experiments without maleimide tempol and EPR spectroscopy albumin with 12 tempols and Hb with two tempols were also generated to map the influence of extension arms and of hexaPEGylation on the superoxide dismutase activity of the conjugated tempol.

Results and Conclusions: EA chemistry-based polynitroxylation is carried out under milder conditions and the thiol maleimide reaction exhibits higher selectivity; it is a better approach than the direct alkylation protocol using bromoacetamido derivative of tempol at higher temperatures. Tempol conjugated through EA chemistry is in a "mobile" configuration vs the "rigid" configuration of tempol conjugated directly onto the protein. The structural of perturbation introduced into the protein core of EAF P5K6 Alb and EAF P5K6-Hb by EAF polynitroxylation is lower than that generated by direct polynitroxylation. In the case of Hb, hexanitroxylation of EAF P5K6 Hb does not influence inter dimeric interactions of PEG-Hb, its molecular radius, and the O_2-affinity. In addition, the SOD mimetic activity of tempol conjugated to Albumin by EA chemistry is twice that of tempol conjugated directly. The antioxidant activity of tempol in Hb is at least six times higher than on albumin. In situations of low or no demand for O_2, as in sickle cell disease or in stroke or traumatic brain injuries, PEG-albumin antioxidants are the preferred macromolecular antioxidants. PEG-Hb antioxidants are the preferred molecules in situations of major hemorrhage.

P-06

STABILITY OF THE MIXTURE OF NITROGLYCERIN (NTG) AND HEMOGLOBIN-BASED OXYGEN CARRIER (HBOC-201) STORED IN PLASTIC BAGS

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Introduction: Our long-term goal is to improve the initial care and ultimate outcome for patients who have suffered polytrauma with traumatic brain injury (TBI) and hemorrhagic shock (HS). The hemoglobin-based oxygen carrier, HBOC-201 (Biopure), has demonstrated survival benefits in animal pre-clinical studies. HBOCs have potential as resuscitation agents as they can improve oxygenation with low volume, but have been associated with adverse events attributable to vasoconstriction. Data from our lab and collaborating investigators have shown that nitroglycerin (NTG) is effective in attenuating systemic and pulmonary blood pressure increases after HBOC-201 administration when co-administered via separate intravascular lines. From a practical view, administration of the drugs as a single mixture would be easier than two separate drugs. Since NTG binds to plastics such as polyvinylchloride (VC), we determined the stability of this combination in oxygen-barrier, double-layer, ethylene-vinyl-alcohol/polyolefin bags for 30 days. In a small pilot study we evaluated whether or not this drug combination, HBOC-201 + NTG, in a single container is stable.
Methods: Tightly sealed aliquots of HBOC and NTG were prepared under nitrogen. Individual bags were prepared for each time point. Mixed samples were stored for 30 days and were analyzed at various times from 0 to 30 days. Mixed solutions with saline were used as controls. Outcomes of stability of HBOC/NTG were defined as: 1) HBOC stability measured by the level of hemoglobin (Hb), Methemoglobin (MetHb), glucose, and lactate; and 2) the level of NTG measured by HPLC of a mixture of 28 μg/ml NTG in 12.5 g/dl HBOC.
Results: The level of NTG in the HBOC/NTG mixture decreased significantly over time, whereas it was stable in the saline/NTG mixtures. The level of Hb in the HBOC/NTG and HBOC/saline mixtures remained stable over time. MetHb was around 6% up to day 1 and slowly decreased in the HBOC/NTG mixture, whereas it remained insignificant in the HBOC/saline mixture.
Conclusions: There could be an adverse reaction occurring between HBOC, NTG, and the bags that makes the HBOC and NTG mixture in non-PVC plastic containers not appropriate for long-term storage. At this point we are not sure if HBOC and NTG mixed together are biologically active. The next step is to extend this study and evaluate if a HBOC-201 and NTG mixture is biologically active.

P-07

IDENTIFICATION OF APPLICATIONS FOR OXYGEN THERAPEUTIC AGENTS (AOTA)

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Problem: It has been proven that oxygen therapeutic agents (OTA) are capable of temporarily substituting the respiratory gas transport capacities of blood. It has been shown that OTAs effectively reduce the transfusion of allogeneic blood units during surgeries with major blood loss. Various clinical trials of OTAs for this particular medical application have been conducted. The major problem remains that the European Medical Association (EMA) will not accept the avoidance of allogeneic blood transfusions as an advance in safety and efficiency, as stated in the annual report 2004 of Alliance Inc., San Diego. Despite their high potential, the impact of OTAs is inhibited due to administrative regulations regarding the licensing process, rather than technical issues. EMA will not allow the comparison between transfusion medicine and alternative methods.
Approach: In order to eventually achieve licensing, modified or completely new applications that are based on the beneficial characteristics of OTAs have to be identified. The Institute of Rescue Engineering (IRE) is pursuing two different strategies: The first approach focuses on the c*omparability* of both the new OTA-based method and the currently used method. Examples are the special requirements of the emergency system, the military, the Third World, religious restraints, regions with low population density, or medical indications (e.g., hemodialysis, leukemia, anemia, tumor oxygenation). The second approach focuses on the *reduction of requirements*, which means the limitation of application specific effects, impacts, and consequently adverse events. Examples are extracorporeal organ perfusion, veterinary medicine, or the cultivation of tissue.
Methodology: The IRE is presently starting a research project for the development of a blood-substitute-based machine perfusion apparatus, called "PerOrgan." Furthermore, the IRE is preparing the research project "AOTA," which is the subject of this paper. In

this project, the following work packages are defined: The identification of processes in which any kind of oxygenation is utilized and analyses of the analogue state-of-the-art practices. Secondly, possible applications of OTAs as alternative methods (as listed above) will be identified, analyzed, and evaluated. A study of safety and efficiency will be processed, prior to the final experimental verification of the results.

Presumption: The IRE is preparing "AOTA" in order to identify possible applications for OTAs, which are likely to have positive results concerning safety and efficiency, and hence licensing by federal authorities such as EMA. It is presumed that a simplification of the licensing application will lead to target-aimed licensing procedures.

P-08

ORGAN HISTOPATHOLOGY AFTER PRE-HOSPITAL RESUSCITATION WITH HEMOGLOBIN-BASED OXYGEN CARRIER 201 AND RECOMBINANT FACTOR VIIa FOR SEVERE HEMORRHAGE IN SWINE

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Background: Previously, this laboratory tested the hypothesis that pre-hospital resuscitation with a resuscitative fluid containing the pro-coagulant recombinant Factor VIIa (rFVIIa) and the oxygen carrier HBOC-201 would improve survival and reduce hemorrhage volume in a swine model of uncontrolled hemorrhage from a liver injury. We identified 90 μg/kg rFVIIa as the optimal dose to administer concurrently with HBOC-201. This combination therapy improved cardiac output and tissue oxygenation during the pre-hospital period compared to HBOC-201 alone. We hypothesized that gross pathology and organ histopathology would also be improved in the 90 μg/kg rFVIIa group.

Materials and Methods: Yorkshire swine were allocated randomly to receive HBOC-201 only or HBOC-201 + various doses of rFVIIa (90, 180, or 360 μg/kg) as pre-hospital resuscitation starting 15 min after injury from grade III liver injury. Animals were monitored for 72 h, euthanized, and evaluated for gross- and histo-pathology.

Results: All groups of HBOC-201 and rFVIIa had effects on lung and liver. Increasing doses of rFVIIa correlated with decreasing incidence/severity of abnormal findings such as pneumonia, pulmonary congestion, and hepatitis. Abnormalities in jejunum, myocardial degeneration, necrosis, and/or fibroplasia were not seen in the 90 μg/kg rFVIIa group, but were observed with increasing frequency with higher rFVIIa doses. No lesions were found in kidney or spleen. Although incidence rates for most findings were higher in the combined HBOC-201 + rFVIIa group compared to the HBOC-201 group, none of the comparisons of incidence/severity between these two groups were statistically significant ($p > 0.05$).

Conclusions: The pathological abnormalities were mainly rFVIIa dose-dependent and 90 μg/kg rFVIIa was the optimal dose for resuscitation with HBOC-201.

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P-09

SYSTEMIC EFFECTS OF SANGUINATE ON TRANSGENIC SICKLE CELL MICE

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Background: Hemoglobin (Hb)-based oxygen carriers (HBOC) have been rarely tested in clinically relevant murine models of sickle cell disease (SCD), although these carriers offer a promising option in the treatment of painful SCD crisis. In addition, carbon monoxide (CO) may stabilize HbS and help in the treatment of SCD. Therefore, we implemented techniques for non-invasive monitoring of physiological parameters in a transgenic SCD mouse model to study the effects of a novel HBOC containing large amounts of CO. We hypothesized that the combination of improved plasma O_2-carrying capacity and the delivery of CO into the microcirculation could be beneficial to Tg SCD mice subjected to a protocol that mimics a vasoocclusive SCD crisis.

Materials and Methods: We used heterozygous Tg SCD "BERK" mice obtained from our colony. After baseline (15 min, 21% O_2), anesthetized mice were subjected to hypoxia (12% O_2, 60 min) followed by reoxygenation (REOX, 50% O_2, 60 min). Sanguinate® (PEG-COHb), obtained from Prolong Pharmaceuticals (South Plainfield, NJ) and prepared from lysed bovine blood (final concentration of 4 g/dl), was given as a top load (8 ml/kg) via a jugular vein 15 min during hypoxia (Protocol 1) or upon reoxygenation (Protocol 2). Core temperature, systemic O_2 saturation (SO_2), heart rate (HR), and respiratory rate (RR) were measured non-invasively. Blood samples were

collected at the end of REOX. Control groups received PEG-Hb, PBS, or bovine Hb.
Results: All mice survived to the administration of all compounds. Changes in SO_2 were similar for all groups. Protocol 1 – all mice showed lowered HR during hypoxia and REOX. The decrease in RR seen in PBS-treated mice was nearly absent in PEG-COHb -treated mice. A 15% decrease in HR during REOX was observed only in PEG-COHb-treated mice. Protocol 2 – all mice showed decreased HR during REOX. In mice receiving PBS, the changes were temporary while the HR of mice receiving exogenous Hb remained below baseline. Changes in RR during hypoxia and REOX were similar among various groups but PEG-COHb treated mice showed the RR values (during REOX) closest to baseline. The final hematocrits of mice treated with PEG-Hb and PEG-COHb were about 14% lower than those measured following PBS and bovine Hb, which may be due to the plasma expansion properties of these PEG-proteins.
Conclusions: Our results suggest that Sanguinate affects heart and respiratory rates but it is well tolerated by Tg SCD mice subjected to hypoxia-reoxygenation. Survival was not affected by treatment in the tested protocols. The significance of the physiological effects deserves further study.
Support: Prolong Pharmaceuticals, Inc., and NIH (BTRP program).

P-10

DESTABILIZING MRNA SECONDARY STRUCTURE NEAR THE TRANSLATIONAL INITIATION REGION ENHANCES PROTEIN EXPRESSION OF ALPHA GLOBIN IN *ESCHERICHIA COLI*

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Background: Extensive attempts have been made to find a suitable alternative to human blood for blood transfusions. Among them, recombinant hemoglobin expressed in *Escherichia coli* (*E. coli*) serves as a great approach for designing a new generation of hemoglobin-based oxygen carriers with fewer side-effects. However, expression of α-globin protein alone has proven more difficult than producing β-globin due partially to rapid protein degradation of unstable apo-α-globin or to a low efficient translation initiation of the α-globin mRNA structure.
Materials and Methods: The complete cDNA clones for human Hb β(MGC:14540), α2 (MGC:14541), and *E. coli* K12 (ATCC:23716) were purchased from the Food Industry Research and Development Institute in Taiwan. Plasmids pET-β and pET-βα were constructed previously. The synonymous codons for the amino acids near the N-terminus of the globin were sequentially chosen based on the highest free energy of the corresponding RNA secondary structure, starting from the transcription start site of pET-15b vector to the code for the specific amino acid of the globin. The free energy was calculated using the MFold method in Seqweb (version 3.1.2, Accelrys, Madison, WI).
Results: The free energies for the synonymous α globin RNA containing 5'-UTR and first 42 nucleotides after start codon were increased to −24.6~−28.4 kcal/mol from -33.1 kcal/mol for the wild-type gene. There was almost no α globin protein expression in *E. coli* BL21 (DE3 ΔclpP::Km) bearing the pET-α plasmid at 37°C. However, the *E. coli* cells transformed with pET-synoα plasmid was able to express α globin protein with, respectively, 1.5 ± 0.5, 2.2 ± 0.6, 2.8 ± 0.80, and $2.24 \pm 0.47 \times 10^{-8}$ μg/cell at 0.5, 1, 3, and 5 h after IPTG induction.
Conclusions: A high stability of α globin mRNA secondary structure near the start codon in the *E. coli* expression system hinders its protein expression. Making silence mutations near the start codon to destabilize the local mRNA secondary structure increases the protein expression level of α globin.

Poster is selected for Young Investigator Award Oral Presentation

P-11

HAPTOGLOBIN GLYCOSYLATION IS ESSENTIAL FOR ITS FUNCTIONAL PROPERTIES

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Background: Several proteins are able to protect against Hb toxicity. One of the most powerful is haptoglobin (Hp). Hp levels in circulation vary greatly among humans, reflecting the fact that Hp is an acute-phase protein. The Hp protein consists of four chains: two α-chains, each about approximately 9 kDa, and two β chains of approximately 33 kDa molecular weights. The beta subunit harbors four carbohydrate chains. The glycosylation pattern of Hp varies, both determined on an individual basis and as an effect of a pathological condition. Different Hp glycosylation profiles are thus associated with the prevalence and clinical evolution of many inflammatory diseases, including infections, atherosclerosis, and autoimmune disorders.
Materials and Methods: Hp was either commercially obtained or produced in E. coli or wheat germ hosts. Deglycosylation of Hp and Hp-HbA complexes were made using PNGase F. pH-sensitive multimodal HIC chromatography was employed to separate different glycan forms of Hp and Hp-Hb. Protein aggregation and stabilities were determined using dynamic light scattering.

Results: The 3D structure of Hp is unknown. However, based on homology modeling a plausible structure has been obtained. Upon Hb binding to Hp, we have shown that these glycan moieties swing out. This can be demonstrated by monitoring enzymatic deglycosylation followed by carbohydrate-specific pH-HIC chromatography. The glycosylation of Hp greatly influences the stability and solubility of the Hb-Hp complex. When fully glycosylated, the complex is extremely stable and even resists boiling conditions.

Glycosylation also affects the ability of Hp to protect against the negative redox reactions caused by Hb. When glycans are removed, this protection is enhanced, as demonstrated by lipid peroxidation studies.

Conclusions: There have been few studies on the role of the Hp glycans on Hb and CD 163 binding, but the carbohydrate moieties are critical for the stability and functional role of Hp.

P-12

APPROACH OF HOLISTIC RESEARCH FOR BIOMEDICAL APPLICATIONS IN THE FIELD OF HAZARD CONTROL AND CIVIL PROTECTION

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Background: The Institute of Rescue Engineering (IRG) of the Cologne University of Applied Sciences was founded in 2010, based on the distinct success in the study program Rescue Engineering (B.Eng and M.Sc.) and interdisciplinary research programs in the field of security research. New scenarios, changing threats, and conflicts force political administrations to react, while demands for solutions are rising. One research field of the IRG is biomedical engineering, especially in the field of disaster, hazards, and emergency scenarios. Combining the disciplines of engineering, natural, social, and business science, economics and civil protection, today's problems and needs can be solved by an interdisciplinary approach.

Methods: Biomedical engineering, in particular, needs multidisciplinary solutions. For this reason the IRG built up a project platform combining different partners of universities, industries, and administrative bodies. Steady scientific exchange of academics, producers, and users assures applied research and hence scientific output. After two years of research in improving the elimination of carbon dioxide within membranes of small, ultra mobile, artificial lungs (ECMO), the IRG started a project in the field of organ perfusion, called PerOrgan. The project contained the supply of organs during the time of explantation by perfusion with blood substitutes. During the prearrangement of this project, several other biomedical projects could be identified.

Results: In addition to the construction of a device for organ annexation and transportation, additional research about an appropriate fluid for perfusion is required. Other interesting projects are biomedical technology used for the adequate management of injured people in mass casualty incidents such as telemedicine documentation and triage, computational analysis of stethoscope data using sensors of artificial intelligence, interface optimization of ambulance services and hospitals, and rheology of blood in different pumps.

Conclusions: New research in biomedical technology requires multidisciplinary approaches. Providing a research platform, the IRG has combined the different competencies of engineering, physiology, telemedicine, disaster management, emergency medical services, organ transport/perfusion, pharmacology, material science, ergonomics, quality management, membrane technology, bio fluid dynamics, and medicine. An exploratory focus will be on the research on blood substitutes and new fields of application.

Poster is selected for Young Investigator Award Oral Presentation

P-13

PULMONARY HYPERTENSION AFTER STORED BLOOD AUTOTRANSFUSION IS PREVENTED BY NITRIC OXIDE BREATHING IN LAMBS

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Background: The pulmonary circulation of both pediatric patients and patients with endothelial dysfunction (ED) is highly sensitive to changes in nitric oxide (NO) bioavailability. During extended storage, red blood cells (RBCs) undergo mechanical and functional changes. These changes increase hemolysis and reduce viability of RBCs, leading to release of hemoglobin, a potent NO-scavenger. We hypothesized that transfusion of RBCs stored for prolonged periods would induce pulmonary vasoconstriction and hypertension in the pediatric pulmonary circulation of lambs, and that ED would markedly increase the vasoconstrictor effect of stored blood transfusion.

Materials and Methods: Based on human blood storage techniques, we developed and validated a new model for autologous transfusion of stored blood in lambs. Ovine leukoreduced RBCs were stored in AS-1 for either 2 or 40

days and transfused autologously over 30 min in awake and instrumented lambs. Pulmonary and systemic hemodynamic parameters were measured continuously during the transfusion and for 4 h thereafter. We also studied the effects of transfusing stored RBCs in lambs after inducing ED by IV infusion of N^G-nitro-L-arginine methyl-ester (L-NAME).

Results: Pulmonary arterial pressure and pulmonary vascular resistance transiently increased during the transfusion of 1 unit of RBCs stored for 40 days (40DS), but not during the transfusion of RBCs stored for two days (2DS). Transfusion of 40DS RBCs did not produce systemic vasoconstriction or hypertension. L-NAME infusion potentiated the pulmonary vasoconstriction and hypertension observed during 40DS RBC transfusion. Concurrent inhalation of 80 parts per million (ppm) NO prevented the pulmonary vasoconstriction induced by transfusing 40DS RBCs. Plasma concentrations of free hemoglobin increased after transfusion of 40DS RBCs, but not after transfusing 2DS RBCs.

Conclusions: Transfusion of 40DS RBCs increased plasma concentrations of free hemoglobin and produced transient pulmonary vasoconstriction and hypertension, but had no effect on the systemic circulation in awake lambs. L-NAME sensitized the pulmonary circulation to the vasoconstriction and hypertension produced by transfusion of 40DS RBCs. Our results imply that free hemoglobin is responsible for pulmonary vasoconstrictor effects after transfusion of 40DS RBCs. The data further suggest that transfusion of stored blood may cause pulmonary vasoconstriction in pediatric patients and patients with endothelial dysfunction, and that supplementing NO may prevent or reverse the pulmonary vasoconstriction.

P-14

POSTIVE ASPECTS OF VASOACTIVITY OF HEMOGLOBIN SOLUTIONS AND THE IMPROVEMENT OF SURVIVAL IN HEMORRHAGIC SHOCK

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Background: Using hemoglobin (Hb) in solution in plasma to provide oxygen-carrying capacity results in the rapid development of hypertension upon introduction into the organism, an effect attributed to the vasoactivity intrinsic to the nitric oxide (NO) scavenging properties of Hb. These phenomena have been associated with the negative outcomes of clinical trials of Hb-based blood substitutes (HBOCs). Induction of hypertension in a normotensive organism is a negative effect, but this is not the case during hypotension, prevalent in hemorrhage. Resuscitation from hemorrhage requires re-establishing blood pressure. In this study we test the hypothesis that vasoactive HBOCs are effective in hemorrhagic shock resuscitation.

Materials and Methods: The HBOC using polymerize bovine Hb (Oxyglobin®, Biopure Inc., 12/.9 g Hb/dl) and the hydroxyethyl starch plasma expander Hextend (HEX, high molecular weight hydroxyl ethyl starch with low degree of substitution, 6%) were compared in terms of survival after exchange transfusion followed by hemorrhage. Studies were carried in the awake hamster window model. Fifty percent blood volume exchange transfusion was followed by a 60% hemorrhage over one hour, followed by one-hour observation. Measurements made were: blood gases, mean arterial blood pressure (MAP), functional capillary density (FCD), arteriolar and venular diameter, and microvascular oxygen tension (pO_2) distribution.

Results and conclusions: Survival with Oxyglobin was 100% with vasoconstriction evident in the microcirculation. Only 50% for the HEX group survived. MAP was higher in the Oxyglobin group. FCD was significantly reduced, although to a lesser extent by Oxyglobin. There was no difference in microvascular pO_2 distribution after one hour of shock between groups.

As in previous studies outcome appears not to be related the distribution of oxygen in the microcirculation, but to the maintenance of FCD. Higher MAP during the initial stages of hemorrhage should be due to vasoconstriction in the Oxyglobin group as compared to the HEX group. The principal effect of the increased MAP should be that of sustaining capillary pressure at near normal levels, a direct consequence of the transmission of central blood pressure to the periphery, suggesting that there is a benefit (in these extreme conditions) in combining low viscosity perfusion with vasoconstriction.

It is concluded that the pressor effect due to a vasoactive HBOC is beneficial in maintaining perfusion when treating conditions of severe hemodilution followed by hypovolemia.

Poster is selected for Tsuchida Prize Oral Presentation

P-15

POINT OF DIMINISHING RETURN: CONTRIBUTION OF TETRAMER CONTENT AND MOLECULAR WEIGHT ON VASOACTIVITY OF HBOC-201

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Background: Hemoglobin-Based Oxygen Carriers (HBOCs) have been under clinical develo

resuscitation for decades. Concern over vasoactivity has hampered further development and numerous studies have shown that reducing low molecular weight (MW) hemoglobin (Hb) content (primarily tetrameric Hb, 65 kD, both unmodified and stabilized) will reduce vasoconstrictive effects. HBOC-201 was developed with reduced tetrameric levels as compared to the veterinary formulation (HBOC-301, Oxyglobin), but to systematically determine if vasoactivity could be further attenuated in HBOC-201, modifications were made both in its tetrameric content and average MW.

Methods: Four HBOCs of varying MW composition were manufactured (OPK Biotech, Cambridge, MA) by glutaraldehyde polymerization of bovine Hb and compared in studies performed by six different laboratories. *In vitro* vascular rings measured vasoactivity via wall tension. Intravital microscopy directly measured vessel diameter in both skeletal muscle and mesenteric vessels in a rat topload model. Studies using systemic and pulmonary blood pressure and vascular resistances as their vasoactive endpoints included two healthy mouse models and two swine models.

Results: Although purification from 35% to 3% low-MW Hb content significantly decreased vasoactive responses, further reduction to $< 0.3\%$ had no additional effect on vasoactivity. Further, increasing the average mw from 400 to 600 kD, while also retaining $< 0.3\%$ tetramer, did not show a difference when compared to HBOC-201.

Conclusions: 1) Tetramer content is not the only determinant of vasoconstriction with HBOCs, as a reduction from 35% to 3% caused a reduction in vasoconstriction but a further reduction to $\leq 0.3\%$ showed no further decrease. 2) Increasing size from average MW of 250 kD to 600 kD also did not decrease vasoactivity of the HBOC. 3) Taken together, these results suggest there are diminishing returns on efforts to eliminate HBOC vasoactivity by further decreasing tetramer content or by modifying polymer size distribution, at least in the 10^2 average MW kD range.

P-16

REVIEW OF PNPH: DESIGN, EFFICACY, AND TOXICITY SUGGEST A PARADIGM CHANGE IN TRANSFUSION AND CRITICAL CARE MEDICINE

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Background: SynZyme's rationale has been to design a hemoglobin-centered nanoparticle therapeutic, a polynitroxylated pegylated hemoglobin in its carboxy form (PNPH). PNPH is multifunctional and takes advantage of the oxygen transport ability of hemoglobin, the anti-oxidative activities from the redox coupling of the covalently labeled nitroxide, i.e. caged nitric oxide (cNO), and heme centers of the hemoglobin, and the hyper-colloid properties of pegylation.

Materials and Methods: PNPH was prepared by polynitroxylation of Prolong's polyethylene glycol conjugated bovine hemoglobin. PNPH had been tested in a model of traumatic brain injury (TBI) complicated by hemorrhagic shock (HS) and in combination with 100% oxygen.

Results: Remarkably, this combination of properties has not only tamed the hemoglobin toxicity, which has been a roadblock to regulatory approval of current generation hemoglobin-based oxygen carriers (HBOCs), but made PNPH neuroprotective. PNPH is most effective in reducing intra-cranial pressure when used with 100% oxygen. In addition, the cNO on PNPH has also been shown to be redox couple with endogenous anti-oxidants, such as ascorbate, to enhance its redox activities.

Conclusion: PNPH as a superoxide dismutase/catalase mimetic nanoparticle provides global protection of endogeneous NO (eNO) levels sensed by the endothelium and the smooth muscle resulting in the correction of inadequate blood flow and prevention of vascular injury from ischemia, reperfusion, and inflammation. Thus, the development of PNPH may represent a paradigm shift in transfusion and critical care medicine beyond volume expansion and oxygen delivery. The filing of an IND for PNPH as a therapeutic resuscitation fluid for TBI complicated by HS is ongoing under a translational research cooperative agreement with NINDS (1U44NS070324 - 01A1).

P-17

MOLECULAR ASPECTS OF *IN VIVO* "ACTIVE SUPRA PERFUSION" BY P5K6 ALB AND P5K6 HB: DEFORMABILITY OF PEG-PROTEIN AND ITS POTENTIAL ROLE IN ENHANCING PERFUSION

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Background: EAF P5K6 Alb and EAF P5K6 Hb have emerged as a new class of plasma expanders that achieve "active supra perfusion." PEGylation induced plasma expander (PE)-like properties of these results in an enhanced perfusion. Moderate vasodilatation and increase in functional capillary densities are seen on the infusion of PEG-Albumin and PEG Hb. These are physiological consequences of high viscogenic plasma expanders like dextran 500, where vasodilatation results from mechano-transduction mediated enhanced endothelial nitric oxide (NO) production. How does EAF P5K6 Alb, a moderately viscogenic solution, mimic the vasodilatory activity of high-viscosity PEs? We advance the hypothesis that this may be due to the uniqueness of their structure as compared to dextran 500. PEGylated Alb and Hb are unique, semi-synthetic hybrid biopolymers that have a compact, globular, high-packing-density

natural biopolymer (protein) and an outer flexible shell of synthetic polymer (PEG) with a low packing density. The lower packing density of PEG endows them with a readily deformable configuration under shear, which induces a differential deformation of the two molecular regions of PEG-protein adducts, enhancing the hydrodynamic volume and hydrostatic drag of the molecule. This increases efficacy of endothelial NO production by PEG Alb and Hb just as high viscosity PEs of uniform packing density. The deformability of the PEG shell should be a function of pattern of PEGylation, presenting the opportunity to manipulate the configuration of PEG-shell to optimize deformability, thus enhancing the stimulus for increasing perfusion, leading to a condition of "active" supra perfusion caused by PEGylated albumin or Hb.

Materials and Methods: PEGylated proteins are generated using extension arm facilitated PEGylation platform. Either albumin or αα-fumaryl Hb is used to produce PxKy-type molecular species, where x represents the molecular mass of PEG (kD) and y the average number of copies of the PEG chains. The present studies are focused to patterns that keep xy at a constant 30K. Four species (P3K10, P5K6, P10K3, and P30K1) have been designed to date.

Results and Conclusions: PEG protein adducts are analyzed for molecular radius, packing density in the PEG shell, viscosity, COP, and deformability as reflected by gel filtration. The PEGylation pattern (P3K10) results in the most compact packing of the PEG shell and the lowest viscosity, COP, and deformability. The P30K1, as the most flexible species, is at the other end of the spectrum. The molecular surface coverage by P10K3 is greater than P30K1, with little difference in deformability between the two species. These PEG Hbs and PEG Albs optimized for "supra perfusion" achieve the synergies of the novel concepts of PEGylation induced deformability, nitrite reductase activity, and enhanced antioxidant activity.

P-18

POLYMER/HEMOGLOBIN ASSEMBLED NANO-PARTICLES AS OXYGEN CARRIERS

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Summary: The cell-like nano-particles were synthesized using the amphiphilic PEG-PLA copolymers after self-assembly. Functional groups were modified onto the side of the PLA chain, via which hemoglobin molecules were conjugated. The system has oxygen-binding ability while changing the oxygen partial pressure and would have great potential to be used as a new type of red blood cell substitute.

Introduction: Blood transfusion is commonly used in clinical trails for saving lives and keeping normal function of the human organs. However, there are many shortcomings in the donated blood; for example, blood type matching is always needed for clinical requirements; the donated blood could only be preserved for three weeks under 4°C; the blood bank is always under shortage; and the most serious problem is virus contamination (such as HIV or hepatitis) of the blood product. Therefore, an artificial blood substitute with high safety, high efficacy, and large-scale production capacity is needed.

In this work, we focus on the simulation of red blood cells. It is well known that hemoglobin (Hb), which is the main protein in red blood cell, carries the main function of transporting oxygen to the human body. The research has revealed that a cell-like shape of the blood substitute may contribute most to the entire system, not only for oxygen transportation, but also for the stability of the blood substitute and the safety of the human circulation system and organs.

Experimental Methods: Amphiphilic triblock copolymers were synthesized using MPEG and cyclic carbonic ester monomer, including propargyl group (MPC) and L-Lactide (LA). These copolymers could self-assemble into core-shell spherical micelles with propargyl groups on the surface. Azided hemoglobin (Hb) was conjugated with the micelles through a click reaction to form Hb-bearing nano-micelles. The click reaction conditions, such as the molar ratio of sodium azide to Hb, the binding ratio of Hb to copolymers, the components of catalytic solution, the reaction temperature and time, were thoroughly investigated and optimized.

Results and Discussion: In this micellar structure, hydrophilic PEG segments serve as the outermost layer to stabilize the micellar structure of PML in aqueous solution; PLA segments form the hydrophobic core; PMPC segments with pendant propargyl groups constitute the middle layer between PEG and PLA parts. The reactive propargyl groups might reside in between the PMPC layer and the PEG layer, making them available for a later conjugation process. As a matter of fact, after mixing with azided hemoglobin, the hemoglobin could be conjugated onto the micellar surface via click reaction between the propargyl and azido groups with a hemoglobin loading ratio of about 70 wt%, which was confirmed by the typical absorbance of hemoglobin in UV and the enlarged micelle size by DLS and ESEM measurement.

The hemoglobin-conjugated PML micelles could bind and release oxygen reversibly with a P50 of 30 Torr, which was proved by the changes of UV absorbance under different oxygen partial pressures. Triblock copolymers containing other groups (for example carboxyl groups) were also synthesized, and the related characterization is still ongoing.

Conclusion: The advantage of these hemoglobin-conjugated biodegradable micelles is obvious, because conjugation between hemoglobin and the micelles results in immobilization of hemoglobin molecules inside the micelles. The hemoglobin molecules are protected by the PEG corona from the attack of the immunological

systems on one hand, and they keep in touch with the aqueous medium in blood in favor of oxygen binding and release on the other hand. Moreover, the PEG segments can stabilize the micelle particles in aqueous medium and prolong the systematic circulation of the micelles.

P-19

MP4OX IMPROVES OXYGEN CONSUMPTION AND SURVIVAL IN A RAT MODEL OF EXTREME HEMODILUTION

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Background: Cell-free hemoglobin solutions were anticipated to improve oxygen consumption (VO_2) and were positioned originally as "blood replacement fluids." However, further studies demonstrated that inappropriate oxygen release characteristics and vasoconstriction could act to limit oxygen delivery. MP4OX was designed as an oxygen therapeutic agent, formulated at low Hb concentration (4.3 g/dL), high oxygen affinity (P50 = 5 mmHg), and high colloid osmotic pressure (COP = 70 mmHg). The present study was conducted to demonstrate that MP4OX maintains VO_2 in a model of extreme hemodilution.
Materials and Methods: Studies were conducted in a rat model of continuous exchange transfusion (ET) and extreme anemia. Conscious male SD rats (290 - 327 g) received ET (0.5 ml/min × 100 min) with one of the following solutions at random: 1) Hextend; 2) PEG-albumin; 3) MP4OX. PEG-albumin served as a pegylated non-O_2-carrying protein with matched COP. Whole body VO_2 was measured from O_2 content in inlet and outlet air of known flow rate with the rat in a sealed chamber.
Results: Baseline VO_2 was similar in all groups (20 - 25 ml/kg/min). Hemodilution was similar in all groups as reflected by the trajectory of hematocrit decline with time, falling to < 5% after 70 min of ET. Hemodilution with either Hextend or PEG-albumin resulted in a rapid decline of VO_2 (< 4 mL/kg/min) at Hb below 4.2 g/dL, and mortality was 100% in both groups by 80 minutes, with terminal arterial lactate > 21 mmol/L. Hemodilution with MP4OX improved survival (87%), VO_2 (11 ± 4 ml/min/kg), and arterial lactate (8 ± 2 mmol/L) at 160 minutes (1 hour after completion of ET). When compared, in a separate set of studies, with αα-crosslinked Hb (at Hb concentration matched to MP4), VO_2 and survival were significantly greater with MP4OX.
Conclusions: These data demonstrate that MP4OX improves oxygen consumption and limits oxygen debt during extreme hemodilution compared to non-oxygen carrying plasma expanders or another Hb-based solution. The data support the concept that MP4OX, a high-affinity hemoglobin molecule, imparts significant benefit as an oxygen therapeutic agent.

P-20

PHARMACOKINETIC PROPERTIES OF HEMOGLOBIN ENCAPSULATED LIPOSOME (HEMOGLOBIN-VESICLE) IN A HEMORRHAGIC SHOCK RAT MODEL

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Background: Hemoglobin-vesicle (HbV) is an artificial oxygen carrier that encapsulates a concentrated human hemoglobin in lipid vesicles (liposome). Despite having a great deal of effectiveness evaluation data such as formulation test and pharmacological test, the pharmacokinetics of HbV have not been well characterized. In addition, it is well known that pharmacokinetic properties of liposome change in response to both pathological conditions and repeated administration. Therefore, in this study, we investigated whether the pharmacokinetic properties of HbV were affected by single or repeated injection in a rat model of hemorrhagic shock.
Materials and Methods: ^{125}I-HbV, in which the inner hemoglobin was radiolabled with iodine, was prepared by incubation of HbV with Na ^{125}I for 30 min at room temperature. The hemorrhagic shock rat model was prepared by removal of 40% of total blood volume. The hemorrhagic shock rats were resuscitated with ^{125}I-HbV to perform the pharmacokinetic study. In addition, to realize the pharmacokinetic properties of repeated HbV injection, the hemorrhagic shock rats were resuscitated with non-labeled HbV, and subsequently the ^{125}I-HbV was administered at 1 hr, 4 and 7 days after resuscitation. The IgG and IgM against HbV was detected by the methods of ELISA.
Results: The major distributed organs and elimination pathway of HbV did not differ between normal and hemorrhagic shock rats, except the half-life was shorter in the hemorrhagic shock rats in comparison with the normal rats while, at 1 hr after resuscitation by HbV, the pharmacokinetic of HbV did not change by the second dose of ^{125}I-HbV as compared to normal rats. However, four and seven days after resuscitation by HbV, the second dose of ^{125}I-HbV was rapidly cleared from the circulation compared to normal rats. At four days post-first HbV injection, the production of anti-HbV IgM was observed, with enhanced phagocyte activity. On the other hand, at seven days post-first HbV injection, only phagocyte activity enhancement can be observed.
Conclusions: HbV showed good metabolic and excretion profile with hemorrhagic shock conditions. However, the retention in blood was changed in response to both

hemorrhagic shock conditions and repeated administration. Therefore, it is necessary to take this point into consideration when deciding the dosing frequency of a dosage regimen. The results obtained in this study would be useful data for future application of HbV in clinics.

P-21

NITRITE REDUCTASE AND ANHYDRASE ACTIVITY OF PEGYLATED HEMOGLOBIN-BASED OXYGEN CARRIERS

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Background: Despite the advances in hemoglobin (Hb)-based oxygen (O_2) carriers (HBOCs) as blood substitutes, the development of materials that are effective in maintaining blood volume and oxygen delivery remain a priority for emergency care and trauma. Vasoactivity, i.e. vasoconstriction, presumably caused by nitric oxide (NO) scavenging, has been defined as the principal problem associated with HBOCs with low hydrodynamic radius. Conversely, Hb-based material with very large hydrodynamic radius (achieved by surface decoration of the Hb tetramer with water-trapping polymers such polyethylene glycol (PEG) conjugation) have been found vasoinactive in spite of being effective NO scavengers.
Materials and Methods: This study explores possible mechanisms for why PEGylated Hbs are not vasoconstrictive and are actually vasodilatory. These studies use *in vivo* assays to compare the efficacy with which different HBOC's can reverse the vasoconstrictive effects of NO depletion through nitrite-mediated reactions. *In vitro* and *in vivo* measurements are used to clarify which Hb-nitrite reactions are responsible for this effect.
Results: *In vivo* vasoactivity and *in vitro* initial nitrite reductase rates imply that high nitrite reductase activity for PEG-Hbs contributes to the absence of vasoconstriction. However, PEG-Hbs nitrite reductase is only part of the picture, as results indicate that PEG-MetHb, in the presence of nitrite and NO, is a very potent vasodilator, capable of compensating for the NO scavenging of acellular Hb.
Conclusions: Vasodilatory activity of PEG-MetHb in the presence of nitrite and NO implicates the formation of S-nitrosothiols (e.g. S-nitrosoglutathione) as a source of long-lived bioactive NO. Overall, the results are consistent with a model in which PEG-Hb high nitrite reductase activity contributes both reactive Met hemes and NO that participate in a MetHb associated reaction (e.g. nitrite anhydrase), which yields either N_2O_3 or NO^+, both effective nitrosating thiols containing peptides. An important implication of these findings is that under clinical conditions where NO synthase is impaired, as would occur during endothelial dysfunction, transfusion of HBOCs, including those derived from PEG-Hbs, are likely to exacerbate vascular condition unless either NO synthase or low plasma nitrite are resolved.

P-22

EFFECTS OF THE MOLECULAR WEIGHT OF TENSE-STATE POLYMERIZED BOVINE HEMOGLOBIN ON BLOOD PRESSURE AND VASOCONSTRICTION

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Background: Despite recent advances in the design of hemoglobin (Hb)-based oxygen carriers (HBOCs), vasoconstriction, presumably caused by nitric oxide (NO) scavenging, hyperoxygenation and/or extravasation, has been identified as the primary road block hampering commercial development of HBOCs.
Materials and Methods: This study was designed to analyze systemic and microvascular responses to the molecular weight (MW) and plasma concentration of tense (T)-state polymerized bovine Hb (PolybHb) solutions. Experiments were performed using the hamster window chamber model subjected to successive hypervolemic infusions of T-state PolybHb solutions. PolybHb plasma concentrations were evaluated, namely: 0.5, 1.0 and 1.5 g/dl, respectively.
Results: Infusion of PolybHb solutions with MWs above 500 kDa elicited hypertension and vasoconstriction proportional to the plasma concentration and inversely proportional to the PolybHb cross-link density. However, two high MW PolybHb solutions, PolybHb(40:1)high PolybHb(50:1)high, did not elicit vasoconstriction at all concentrations studied, while PolybHb(50:1)high only elicited moderate hypertension at the highest concentration studied. In contrast, infusion of PolybHb solutions with MWs below 500 kDa elicited significant hypertension and vasoconstriction compared to PolybHb solutions with MWs above 500 kDa that was proportional to the plasma concentration and inversely proportional to the PolybHb cross-link density.
Conclusions: This study presents promising results for highly cross-linked T-state PolybHb solutions with MWs above 500 kDa (PolybHb(40:1)high PolybHb(50:1)high), which supports the concept that HBOC size/molecular weight influences its proximity to the vascular endothelium and molecular diffusivity. We hypothesize that the hemodynamics of HBOC within the plasma layer surrounding the abluminal side endothelium regulates NO production and consumption, oxygen flux to the blood vessel wall, and extravasation through the blood vessel wall. Although mechanistically attractive, neither of these

hypotheses can be directly tested *in vivo*, and will require further investigation.

P-23

VASOACTIVE AND OXYGEN TRANSPORT DIFFERENCES OF POLYMERIZED BOVINE HEMOGLOBIN: OXYGLOBIN VS HEMOPURE

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Background: Hemoglobin (Hb) oxygen (O_2) carriers (HBOCs) are potential alternatives to blood transfusion when native O_2 carrying capacity is lacking. OPK Biotech LLC has two O_2 therapeutic products based on polymerized bovine Hb, Hemopure™ (HBOC-201) and Oxyglobin™ (HBOC-301). They have similar O_2-carrying properties, but different colloid oncotic pressure (COP) and pharmacokinetics due to differences in the final tetrameric versus polymer content.
Study Design and Methods: The objective of this study was to determine differences in microvascular vasoactivity, organ perfusion, and tissue O_2 transport between HBOC-201 and HBOC-301. The physiological changes to HBOC-201 and HBOC-301 were studied during extreme anemia (11% hematocrit) in the hamster window chamber model. Anemic conditions were induced by hemodilution with a plasma expander (6% dextran 70 kDa). Animals were randomly assigned to exchange transfusion groups of the O_2 therapeutic product and its concentration, namely: HBOC-201 and HBOC-301 at 13gHb/dl [Hemo-13 and Oxy-13], and 8gHb/dl [Hemo-8 and Oxy-8] in albumin solution to match COP. The control group was exchange transfused albumin solution also at matching COP [Hemo-0 and Oxy-0]. Groups were analyzed based on measurements of systemic parameters, organ blood flow, microvascular hemodynamics, capillary perfusion, and O_2 levels.
Results: Mean arterial pressure (MAP) for the Hemo-13 and Oxy-13 groups were not different from baseline. Hemo-13 had significantly lower MAP compared to Oxy-13. Hemo-8 and Oxy-8 showed lower MAP compared to baseline. Hemo-8 had significantly lower MAP compared to Oxy-8. Hemo-0 and Oxy-0 had lower MAP compared to baseline and the groups hemodiluted with HBOC formulations. Microvascular vasoconstriction increased with the concentration of acellular Hb; however, vasoconstriction was lower for HBOC-201 compared to HBOC-301. Analogous to the changes in diameter were their effects on microvascular blood flows. Hemo-13 and Hemo-8 sustained significant higher tissue oxygen than Hemo-0, and Oxy-8 also had significant higher tissue oxygen compared to Oxy-0. Even though the O_2 affinities are similar, Hemo-13 and Hemo-8 produced higher tissue O_2 compared to Oxy-13 and Oxy-8. The microcirculation perfusion changes correlated with the blood flow distribution to vital organs including the kidneys, brain, and heart.
Conclusion: In summary, these results indicate there are differences between clinical (HBOC-201) and veterinarian (HBOC-301) formulations in terms of vasoactivity, organ perfusion, and oxygenation. The importance of increasing plasma O_2-carrying capacity during extreme anemic conditions was established with both formulations. The optimal amount of HBOC should be set based on functional markers including pressure, perfusion, microvascular function, and oxygenation, leaving behind the old paradigm of concentration or units.

P-24

NORMALIZATION OF HBOC-INDUCED VASOCONSTRICTION BY SIMULTANEOUS VASODILATOR ADMINISTRATION IN CONSCIOUS, INSTRUMENTED SWINE

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Background: Most hemoglobin-based oxygen carriers (HBOCs) sequester nitric oxide, resulting in vasoconstriction, elevated blood pressure (BP), and decreased cardiac output (CO). BP increases following HBOC-201 are usually transient and mild. Associated decreases in CO are normal adaptations to increased cardiac afterload. No hard evidence links vasoactivity to a safety imbalance in HBOC-201 clinical trials. Preclinical data demonstrate maintenance of blood flow to and oxygenation of brain, heart, and kidney and clinical studies indicate normal coronary blood flow (CBF) during HBOC-201 administration to patients. However, some still perceive a need to minimize HBOC vasoactivity. Study aim: Explore strategies to reduce HBOC-201 vasoactivity.
Materials and Methods: Swine were chronically instrumented for pulmonary artery pressure (PAP), aortic pressure (AP), CO, CBF, and heart rate (HR) in awake animals. One week after surgery, swine were studied before HBOC-201 at rest and at five levels of treadmill exercise (1,2,3,4,5 kg/h, 2-3 min/level), resulting in HR = 85% of max. Hemodynamics were recorded continuously, arterial and coronary venous blood (pH, lactate) was collected, and myocardial lactate consumption determined. The baseline exercise program was followed by 30 min of rest and infusion of HBOC-201 (1.3 g/kg) or HBOC-201 + intravenous vasodilator nitroglycerin (NTG) or adenosine (ADO) to maintain pre-HBOC MAP ± 5 mmHg. The exercise program was then repeated.
Results: CO was proportional to exercise intensity up to 2-fold above CO at rest due to increased HR. CBF increased with increasing exercise by up to 80%. Compared to control, HBOC-201 increased MAP (by 27mmHg), PAP (by 11mmHg), systemic vascular resistance (SVR, by 47%), and pulmonary vascular

resistance (PVR, by 69%) at rest. Neither HBOC-201 or HBOC + vasodilator altered myocardial work (MW) at rest or during exercise. Following HBOC-201, CO was similar to control at rest and slightly lower than control during exercise. Although HBOC-201 increased coronary vascular resistance (CVR) by 22-24% during rest and exercise, CBF was similar to control. There was no indication of anaerobic myocardial metabolism before or after HBOC-201. NTG or ADO eliminated HBOC-induced decrease in CO and increases in SVR and PVR. However, NTG, but not ADO, eliminated the HBOC-induced increase in PAP.

Conclusions: Despite HBOC-201-induced increases in MAP and PAP, MW and myocardial metabolism were unaltered by HBOC-201, even during strenuous exercise. The HBOC-201-induced increases in MAP, PAP, SVR and PVR can be prevented via simultaneous infusion of NTG or ADO.

P-25

BINDING AND REDOX REACTION KINETICS OF FREE AND CHEMICALLY MODIFIED HEMOGLOBINS WITH HAPTOGLOBIN

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Background: The abundant plasma protein haptoglobin (Hp) binds to hemoglobin (Hb) with high affinity and plays a major role in the clearance of cell-free Hb after hemolysis. Hp has long been recognized to bind primarily Hb dimers but not tetrameric forms. We report here the fluorescent rapid mixing and redox reaction kinetics of free and some chemically modified Hbs with Hp and the relevance of these observations to *in vivo* oxidative inactivation and clearance of Hb-based oxygen carriers (HBOCs).

Materials and Methods: We carried out equilibrium binding, rapid kinetics, and spectroelectochemical experiments to further investigate the molecular mechanism of Hp and Hb interaction and its effects on Hb ligand and redox reactions. Hb tetramers with two alpha subunits or two beta subunits specifically cross-linked, respectively, were analyzed and compared with the native Hb.

Results: Our kinetic measurements revealed that ββ cross-linked Hb binds to Hp with lower affinity than that of native Hb, whereas the αα cross-linked Hb showed little or no binding activities. Under equilibrium conditions, the binding of Hp increased oxygen-binding affinity, by left shifting of the oxygen equilibrium curve. This is supported by the oxygen dissociation and CO association rate constants measured in the stopped-flow instrument. Hp also accelerated the oxidative reactions of native Hb with nitrite and stabilized the ferryl Hb formed in the presence of H_2O_2. In contrast, Hp had no effects on the reactions of ββ cross-linked Hb with these ligands. Similarly, no differences were seen in the redox reactions of native and modified Hbs, including autoxidation, metHb oxidation by H_2O_2, NO dioxygenation, ferryl Hb stabilization ,and heme degradation by H_2O_2, in the presence or absence of Hp. Interestingly, the spectroelectrochemistry experiments on native Hb and ββ cross-linked Hb bound to Hp showed a shift of the reduction potentials towards more negative values by 70 and 54 mV, respectively, [vs. normal hydrogen electrode] with respect to the redox values of free Hb and ββ cross-linked Hb under identical conditions. These shifts of the reduction potentials towards lower potential values indicate the stabilization of the higher oxidation state in the complexed form.

Conclusions: Studies reported here clearly show that Hp can be used in the clearance and oxidative inactivation of native Hb and ββ cross-linked Hb. It may prove necessary to explore these protective clearing mechanisms to counter the toxicity associated with some oxygen therapeutics and with free Hb in hemolytic anemias.

P-26

HEMOGLOBIN-NANOPARTICLES AS POTENTIAL OXYGEN CARRIERS: SYNTHESIS AND PROPERTIES

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Background: The search for a safer blood substitute has resulted in the identification of properties that are needed to minimize previously encountered side-effects with HBOCs. These properties include increased molecular weight to minimize vasoconstriction and incorporation of catalase and superoxide dismutase (SOD) to decrease oxidative damage.

Materials and Methods: Bovine hemoglobin was cross-linked between the β82 lysines using bis(3,5-dibromosalicyl) fumarate (bb82XLHb).The reactive groups for were complementary chemistry introduced into different proteins using N-γ-maleimidobutyryloxy-succinimide and Traut's reagent, and the proteins were mixed to produce the polymer. These polymers have been characterized with respect to size, oxygen binding, and autoxidation.

Results: We have made high-molecular-weight polymers of Hb alone and of Hb with either or both catalase and SOD. There is a decrease in both the p50 and Hill coefficient for all polymerized species. The polymers containing catalase and/or SOD had smaller autoxidation rates than Hb-only polymers. These are, however, amorphous polymers that are not uniform in size.

Producing approximately spherical nanoparticles of these materials may improve their properties.
Conclusions: The keys for success in forming nanoparticles are the degree of modification on the proteins, the way in which the reactive components are mixed and reacted and, finally, rapid separation of the product from the reactants. By using repeated cycles of reaction we are able to add layers of protein to the particles and, thus, control the size of the nanoparticles. With larger preparations of the nanoparticles we will test them in a rat hypovolemic shock model.

P-27

HEMOGLOBIN PHARMACOLOGICALLY MODIFIED WITH ATP, ADENOSINE, AND REDUCED GLUTATHIONE: CURRENT DEVELOPMENT STATUS

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Background: Although Hb-based oxygen carriers may offer a solution to transfusion medicine problems such as blood shortages, transmission of bloodborne pathogens and the RBC storage lesion, all commercialization attempts to date have been unsuccessful due to efficacy or toxicity issues. We have developed HemoTech, the next-generation blood substitute that utilizes the concept of "pharmacologic cross-linking."
Materials and Methods: HemoTech, which consists of bovine Hb cross-linked intramolecularly with *o*-ATP and intermolecularly with *o*-adenosine, and conjugated with reduced glutathione (GSH), has entered the regulatory process in the USA. Several mandated requirements have been met, including viral and prion clearance validation studies performed by BioReliance (Rockville, MD, USA) and various non-clinical pharmacology, toxicology, genotoxicity, and efficacy tests conducted at the Research Toxicology Centre (Pompezia, Italy). The effects of HemoTech on appropriate physiological measures in human cell systems, normal animals, and disease models have also been determined. HemoTech was tested *ex vivo* on human platelets obtained from percutaneous coronary intervention (PCI) patients. The clinical proof-of-concept was carried out by the Instituto Sierovaccinogeno Italiano (S. Antimo, Italy).
Results: In this composition, while ATP prevents Hb dimerization, adenosine permits the formation of homogeneous polymers and counteracts the vasoconstrictive and pro-inflammatory properties of Hb via stimulation of adenosine A_2 and A_3 receptors. ATP also serves as a regulator of blood vessel tone through activation of the P2Y receptor. GSH introduces electronegative charge onto the Hb surface that blocks Hb's transglomerular and transendothelial passage and shields heme from nitric oxide and reactive oxygen species, thus enhancing vasodilation and lowering Hb's pro-oxidative potential. The results of preclinical and clinical studies indicate that HemoTech can work as a physiological oxygen carrier with prolonged intravascular persistence and produces no adverse nephrotoxic, neurotoxic, oxidative, or inflammatory reactions. It has vasodilatory activity as well as high erythropoietic potential. It was found that HemoTech decreases platelet aggregability in response to platelet aggregation agonists, particularly collagen, and blocks the release of serotonin. Based on this observation and previous studies, it is believed that HemoTech has the potential to mitigate myocardial ischemia and thrombotic events associated with PCI.
Conclusions: The obtained results confirmed that "pharmacologic cross-linking" of Hb molecules with ATP, adenosine, and GSH is highly effective in designing a viable blood substitute.

P-28

ORTHOGONAL METHOD FOR THE REMOVAL OF PRIONS AND VIRUSES FROM HEMOGLOBIN SOLUTION

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Background: Hb solutions of human and bovine origin, to be effective oxygen-carrying plasma expanders, must fulfill a number of requirements. In addition to being non-toxic, non-immunogenic, non-pyrogenic, having an extended shelf-life and a satisfactory oxygen-carrying capacity, these products should be entirely pathogen-free. This includes being free of prions of human and bovine origin that cause Creutzfeldt-Jakob Disease (CJD), Bovine Spongiform Encephalopathy (BSE), and viruses. The manufacturing process for human- and animal-derived products must be validated for its capacity to clear pathogens by at least two major and independent (orthogonal) clearance steps. One of the clearance steps must be inactivation and the other step could be removal.
Materials and Methods: We have developed a prion/viral clearance platform that is an integrated orthogonal concept for robust and reliable clearance of these pathogens from Hb solutions. This orthogonal multi-step procedure comprises: (i) nano-filtration; (ii) membrane chromatography; (iii) solvent treatment; and (iv) heat inactivation. The offered technology can be used as a separate entity or be incorporated into existing manufacturing processes to ensure the absence of these pathogens in the final product. The clearance validation tests have been performed by BioReliance/Invitrogen Laboratories (Rockville, MD) using bovine Hb spiked with prions and viruses; enveloped (Bovine Viral Diarrhea Virus – BVDV, Leukemia Virus C-Type Retrovirus - X-MuLV, Bovine Rhinotracheitis

Virus – IBR) and non-enveloped (Encephalomyocarditis Virus – EMCV, Bovine Parvo Virus - BPV).
Results: The clearance validation tests confirmed that this orthogonal technology is extremely effective in the elimination of prions and enveloped (BVDV, X-MuLV, IBR) and non-enveloped viruses (EMCV, BPV). While the clearance of viruses was, on average, 1 - 4 log10 reduction value (LRV) above the FDA limits, the prion elimination was more than 10 LRVs, exceeding the FDA requirements by 5 LRVs.
Conclusions: The advanced nature of the proposed technology is to combine the orthogonal capability of this method with simultaneous clearance of diverse pathogens, ranging from proteins to nucleic acids. This patented technology is fully operational, validated, and consistent with any batch size, and could be used for other therapeutics derived from human and bovine sources.

P-29

EXTRACELLULAR HEMOGLOBIN ATTENUATES NITRITE-ENHANCED ATP SYNTHESIS AND RELEASE FROM RED BLOOD CELLS

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Background: There is still controversy regarding the exact mechanism by which extracellular Hb causes vasoconstriction. Neither currently proposed mechanisms nor previous theories to explain this phenomenon have resulted in the development of an effective preventive strategy. The concept of using the nitrite reductase activity of Hb that generates nitric oxide (NO) equivalents to treat unwanted hemodynamic side-effects has proven to be less clinically promising than anticipated. Recently, it has been established that nitrite enhances the release of ATP from RBCs, which induces vasodilation by stimulating the P2Y-purinergic receptor on the endothelium that is linked to the production of nitric oxide. Since the oxygen affinity of Hb can modify the erythrocytic ATP release and Hb is highly reactive with nitrite, this study was undertaken to investigate the impact of extracellular Hb on nitrite-induced ATP synthesis and release from RBCs.
Materials and Methods: Fresh human RBCs suspended in isotonic PBS buffer (10% Hct, pH 7.4) were exposed to purified bovine Hb (1.3 g%) and sodium nitrite (500 uM), under hypoxic and normoxic conditions. After one hour of incubation at 37°C, RBCs were evaluated for intracellular ATP and supernatants were screened for ATP, nitrite, and ferric Hb. ATP was measured by the luminescence method using Chrono-Lume Luciferase-Luciferin reagent and Lumi-Aggregometer (Chrono-Log Corp., Havertown, PA). Hb oxidation was assessed with IL682 CO-Oximeter (Instrumentation Lab., Clayton, NC) and decay of extracellular nitrite with Griess reagent (Cayman Chemical Co., Ann Arbor, MI). All experiments were performed in triplicate. Data was subjected for statistical analysis using StatWorks software.
Results: As expected, nitrite stimulated ATP synthesis and released from RBCs, more in hypoxic than normoxic conditions. Extracellular Hb diminished the effect of nitrite in both oxygen environments. A rapid consumption of extracellular nitrite was associated with formation of ferric Hb.
Conclusions: The nitrite-induced ATP synthesis and release from RBCs can be suppressed by extracellular Hb, which may contribute to Hb-mediated vasoconstriction.

P-30

ENHANCED NITRITE REDUCTASE ACTIVITY OF MP4OX, A NOVEL HEMOGLOBIN-BASED OXYGEN THERAPEUTIC AGENT

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Background: Hemoglobin-based oxygen carriers have long been associated with vasoconstriction that has been attributed to nitric oxide (NO) scavenging by heme. MP4OX, an oxygen therapeutic agent, designed using poly(ethylene glycol) (PEG) conjugation chemistry, has been observed not to cause vasoconstriction and to preserve microvascular blood flow. Recently, evidence has been presented that reduction of nitrite to NO by deoxyhemoglobin has the ability to vasodilate blood vessels. This nitrite reductase activity of hemoglobin is under allosteric control and produces NO at a maximal rate when deoxyhemes are in an R-state conformation. Based on work done with PEG-hemoglobins by Kluger and coworkers, we hypothesized that MP4OX, which is in a stabilized R-state conformation, should have enhanced nitrite reductase activity, which may contribute to its lack of vasoconstriction.
Materials and Methods: MP4OX was prepared from the reaction of stroma-free hemoglobin (SFH) with 2-iminothiolane and maleimide-activated PEG to produce a PEG-hemoglobin conjugate. Deoxygenated SFH and MP4OX were reacted anaerobically with sodium nitrite in a sealed cuvette in the presence of sodium dithionite. The reaction was monitored spectrophotometrically at various concentrations of excess nitrite. The resulting spectral data were deconvoluted using parent spectra for deoxyhemoglobin, iron-nitrosyl-hemoglobin, and methemoglobin. Since hemoglobin species can deviate from pseudo first-order kinetics for this reaction due to T-to-R state allosteric transition, rate constants were derived from the disappearance of deoxyhemoglobin during the initial phase of the reaction kinetics.

Results: The reaction rates of SFH and MP4OX with excess nitrite were linear with nitrite concentration. Analyses of the time courses showed that both reactions had autocatalytic properties. SFH deviated substantially from pseudo first-order kinetics, as expected due to its allosteric transition, while MP4OX exhibited only minor cooperativity. SFH and MP4OX reduced nitrite to NO with initial rate constants of 0.13 $M^{-1}s^{-1}$ and 3.6 $M^{-1}s^{-1}$, respectively, showing a 27-fold higher rate for MP4OX compared to SFH.
Conclusions: Our results show that MP4OX is more effective at reducing nitrite to NO than SFH, which may compensate for NO scavenging and explain, at least partly, why MP4OX does not induce vasoconstriction in vivo. The increased reaction rate of MP4OX appears to be due to R-state stabilization, at least partially resulting from PEG conjugation at the βCys93 sites. Further studies are required to understand the extent to which MP4OX's enhanced nitrite reductase activity contributes to its ability to improve perfusion and oxygenation of ischemic tissues and open the vasculature for targeted oxygen delivery in the capillaries.

P-31

INFLUENCE OF HEMOGLOBIN VESICLE ON OXYGEN DIFFUSION CONSTANT OF ARTERIOLAR WALL IN MICROCIRCULATION OF MOUSE DORSAL SKIN WINDOW CHAMBER

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Background: Oxygen transport to tissues is thought to carry out through the capillary. However, oxygen saturation of hemoglobin in arteriolar blood decreases with branching. Several studies have reported that oxygen diffuses through arterioles to tissues. Meanwhile, an artificial oxygen carrier is reported to interact with endothelium and perivascular smooth muscle cells through nitric oxide signaling. It is important to clarify the change of oxygen diffusion constant of the arteriolar wall after the artificial oxygen carrier.
Materials and Methods: We have set up a unique microscope system which can measure tissue oxygen tension simultaneously at the different spot using a laser (23 micrometer diameter) excited Pd-phosphorescence quenching method. This microscope system enabled temporal change of PtO_2 after inhalation of pure oxygen.

Subcutaneous microcirculation was visualized using a mouse dorsal skin window chamber (n = 4, sites of arteriole = 12), which was implanted 2-6 days before experiment. Anesthesia was induced by intramuscular Ketamine-Xylazine cocktail. An intravenous needle (30G) was inserted in the tail vein and fixed. 2-2.5%Sevoflurane was used for maintenance. Pd-coproporphirine was injected 30 minutes before experiment. The dorsal skin window chamber was fixed with double-sided tape to a Piezo-driven stage. This stage moved horizontally up to 100 micrometers at a rate of 5 Hz; thus we can measure PtO_2 at two different spots (inner and outer edge of arteriolar wall) simultaneously.

After stabilization, a 40-80 micrometer inner diameter arteriole was identified and measurement of diameter of arteriole and arteriolar wall thickness was done. Then we started to measure PtO_2. Pure oxygen was inhaled and the change of tissue oxygen tension was recorded. We can observe the increase of oxygen tension at the inner and outer edges of the arteriolar wall. A difference of time when PtO_2 rose was identified and stable oxygen tension after oxygen inhalation was read from the record.

After measurement of control state, HbV (10g.dl Hb concentration, suspended in saline) were administered slowly (20% of estimated blood volume). Ten minutes after administration of HbV, another set of measurements was done. Subsequently, the oxygen diffusion constant was calculated.
Results and Summary: Kroh's oxygen diffusion constant of arteriolar wall was 6.60 + /−1.57E-11 (cm^2/s) (ml $O_2 \cdot cm^{-3}$ tissue·$mmHg^{-1}$) in control state and 8.33 + /−3.32E-11 (cm^2/s) (ml $O_2 \cdot cm^{-3}$ tissue·$mmHg^{-1}$) after 20% top load of HbV. There is no significant difference. HbV showed inert activity in regards to the oxygen diffusion through the arteriolar wall.

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DESIGN, SYNTHESIS, AND ASSEMBLY OF AMPHIPHILIC AND BIODEGRADABLE BLOCK COPOLYMERS FOR ENCAPSULATION OF HEMOGLOBINS

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Background: Among the red blood cell substitutes, polymeric micelles and vesicles are expected to have much better mechanical strength and blood stability than liposomes. Chang and co-workers used PEG-PLA copolymers as the membrane of the Hb-vesicles to prolong the circulation time, to facilitate the inclusion of enzyme systems, and to avoid direct exposure of Hbs to plasma. In our group, preliminary results were obtained on Hb-micelles and Hb-vesicles. In order to optimize the carrier polymers, several series of biodegradable block copolymers were designed and synthesized. Their assembly behaviors without and with Hbs were examined.
Materials and Methods: All amino acids were converted to corresponding N-carboxy-anhydrides (NCAs) or protected NCAs, and were polymerized with a certain amino compound as initiator. Poly(lactic acid) (PLA) or poly(ε-caprolactone) (PCL) was prepared by ring-opening polymerization of L-lactide or ε-caprolactone with an

alcohol or a poly(ethylene glycol) (PEG) as initiator and with diethyl zinc or stannous octanoate as catalyst. Functionalized carbonate monomers were synthesized in our own laboratory and were copolymerized with L-lactide or ε-caprolactone. The molecular structures of the copolymers prepared were characterized with FT-IR, ^{1}H NMR, and GPC. The polymers were assembled into nanoparticles or vesicles by direct emulsification or dialysis.

Results: Several series of copolymers were prepared. They were: (1) poly(L-lysine)-b-poly(phenylalaline) (PLL-b-PPA), poly(L-lysine)-b-poly(L-cystine)-b-poly (L-phenylalaline) (PLL-b- PCy-b-PPA), and PLL-b-P(PCy-co-PPA); (2) PEG-b-PCL-b-PLL; (3) poly((L-lactide)-co- (2,2diazidomethylpropylene carbonate))-graft-PEG; (4) poly(ethyleneglycol)-b-poly(L-lactide-co2-methyl-2-trimethoxysilyl-propyl-thia-propyloxycarbonyl-propylene carbonate); and (5) Y-shaped copolymer poly (L-glutamic acid)-b-poly(L-lactide)$_2$ (PLGA-b-(PLA)$_2$), etc. Their molecular weights and length of each block were measured. Self-assembling was performed. Aggregate morphologies (micelles and vesicles) and particle sizes were observed and measured by SEM, TEM, or DLS and were correlated to molecular structures and the relative lengths of the constituent blocks.

Conclusions: Biodegradable amphiphilic block copolymers with proper structures may self-assemble into polymeric vesicles with or without hemoglobin inside. Therefore, they may be used as the carriers for hemoglobin encapsulation. Crosslinking may be introduced to the vesicles formed to further improve their stability and strengths.

P-33

USE OF FETAL HEMOGLOBIN (HBF) AS AN EFFECTIVE PLATFORM FOR PRODUCING BLOOD SUBSTITUTES

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Background: Fetal hemoglobin (HbF) is the main oxygen transport protein in the fetus during the last seven months of development in the uterus and in the newborn until roughly six months old. Some functional properties of HbF, such as its oxygen affinity and its interaction with allosteric regulators such as 2,3-DPG, differ considerably from the corresponding values of HbA.

Hb has a rich chemistry associated with the free radical reactivity of its heme group. There is a growing awareness that Hb easily can become a toxic molecule in itself. Hb can react with free radical signalling molecules like nitric oxide, but it can also create free radicals when challenged by peroxides under conditions of oxidative stress. First, the ferrous iron in the oxyhemoglobin molecule spontaneously autoxidizes, producing the ferric (met) protein. The met protein can then further react with peroxides, generating a reactive ferryl iron and free radicals bound to the protein. Circulating Hb released from damaged red blood cells or alternatively from Hb-based oxygen carriers (HBOC)s is thus highly susceptible to heme iron, heme, and hemeprotein oxidation when exposed to oxidative environments, despite well-operating antioxidant and clearance mechanisms.

In the present study we have examined some of the key properties of HbF essential for designing a functional blood substitute, including its production and purification, redox properties, stability, binding to haptoglobin (Hp), and detoxicification possibilities using e.g. alfa-1-microglobulin (A1M).

Materials and Methods: HbF was obtained from co-expression of the chains using a pETDUET vector system in *E. coli*. HbF was also purified from cord blood samples. A1M was isolated from urine.

Results: Since HbF is present almost exclusively as a tetramer, purification from E. coli extracts therefore becomes greatly facilitated as compared to HbA. In analogy with HbA, HbF reacts with the oxidant H_2O_2 to form a higher oxidation ferrylHb on the heme group and an associated oxidized globin amino acid free radical. However, when compared to HbA, HbF shows reduced intrinsic redox reaction rates. These oxidative effects caused by addition of excessive amounts of H_2O_2 can also be completely attenuated by addition of Hp by forming a stable HbF–Hp complex. Alternatively, supplementing minor amounts of A1M can effectively block negative redox reactions caused by HbF.

Conclusions: HbF is a more robust Hb alternative compared to HbA when designing blood substitutes. It is also much less prone to dimer formation. HbF binds avidly to Hp and this complex may be explored to develop a simple blood substitute, since negative redox reactions can be effectively blocked. A1M can be explored as an effective antioxidant in vesicle-based HBOCs.

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WHOLE-BODY ASSESSMENT OF HBOC-RELATED ADVERSE EFFECTS BY NANOSPECT/CT TECHNOLOGY IN RAT, GUINEA PIG, AND MOUSE

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Background: Adverse effects (AEs) due to nitric oxide (NO) scavenging have been shown to hamper the therapeutical potential of hemoglobin(Hb)-based oxygen carrier (HBOC) as a blood substitute, because reduced bioavailability of NO

(RBNO) can lead to pathologies such as vasoconstriction, red blood cell (RBC) and platelet aggregation, which can develop in an organ-specific manner. Hence the need for whole-body assessment of AEs of HBOCs both in the basic and pre-clinical phases of research.

Materials and Methods: A 6% hydroxyethyl starch solution (HES, Voluven®) was used as negative control. Euro-PEG-Hb (EuroBloodSubstitutes) was produced in a concentration of 7 g% and was given to anesthetized rats and guinea pigs as 50% blood-to-blood substitute exchange. RBNO in absence of HBOC was produced by an I.V. injection (100 mg/kg bdw, i.v.) of N-nitro-L-arginine methyl-esther (L-NAME) in anesthetized rats, guinea pigs, and mice. RBCs were labeled with ^{99m}Tc using stannous pyrophosphate (PyroScint®) as reducing agent (20 µg Sn (II)/kg bdw, i.v.). Thirty minutes later, 1 mL of pre-treated arterial blood was withdrawn and mixed with 1 mL of ^{99m}Tc-pertechnetate solution of ~ 200 MBq activity and allowed to stand for 10 minutes prior to re-injection. Labeled RBCs were re-injected (in 0.7 mL of ~ 70 MBq activity) for mapping RBC mass 5 minutes post-injection. Whole-body helical SPECT/CT scans (NanoSPECT/CT equipment, Mediso Ltd.) were acquired for control and at one hour, one day, and one week. RBC mass was mapped normalized by activities in the heart chambers using the InVivoScope software (Bioscan, USA).

Results: As compared to animals treated with HES, our exemplary experiments demonstrated markedly elevated levels of RBC mass at one hour in the lungs, both in rats and guinea pigs which underwent blood-to-HBOC exchange. Similar increase could be produced by L-NAME, not only in rat and guinea pig but in mouse, too. The RBC mass in the lungs of HBOC-treated guinea pig remained elevated at one day and one week.

Conclusions: Employing small animal SPECT/CT technology in rodents, we demonstrated a strong and lasting AE of HBOC treatment in the lungs due to RBNO. Prospectively, this technology could prove effective in pre-clinical screening for AEs of HBOC products, as the impact of RBNO on the lungs was seen in mouse that would permit simultaneous scanning of several of this small-size animal in a single session.

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COULD HBOC IN THE PRESENCE OF ENDOTHELIAL DYSFUNCTION BE BAD FOR YOU? A HYPOTHESIS

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Background: Clinical trials using Hemoglobin-based Oxygen Carriers (HBOCs) as transfusion replacement in subjects undergoing elective surgical procedures have shown some adverse events occurring with greater frequency than those in control subjects receiving blood transfusions. The majority of subjects undergoing these elective surgeries have been elderly in whom hypertension, atherosclerosis, and diabetes are highly prevalent. These latter conditions are characterized by *endothelial dysfunction* (ED) manifested in: (1) reduced NO production in response to normal agonists; (2) excessive endothelin production in response to normal agonists; (3) vasoregulation favoring vasoconstriction; and (4) excess generation of *reactive oxygen species* (ROS).

The evidence: Cell-free hemoglobin (Hb) gains easy access to the sub-endothelium where it scavenges the locally generated nitric oxide (NO) avidly, thereby reducing the bioavailability of NO for its normal target soluble guanosyl cyclase (sGC), generating cyclic guanosyl monophosphate (cGMP), which in its turn inhibits cross-bridge cycling in vascular smooth muscle cells (VSMC), resulting in vasodilation. Concurrently, the unprotected Hb may undergo changes that favor the generation of ROS, which react avidly with NO to form peroxynitrite ($ONOO^{\cdot}$), further reducing NO bioavailability. Free Hb also induces the release of endothelin.

Endothelial dysfunction: In ED normal vasodilation in response to normal stimuli is replaced by vasoconstriction, due to an imbalance in NO/endothelin response favoring the latter. Furthermore, ROS are generated in excess, with reduction in the protective enzymatic activities capable of scavenging the excess ROS. This excess of ROS inhibits eNOS, reacts avidly with NO, and inhibits sGC. The dysfunctional endothelium is also more permeable to macromolecules, thereby permitting greater direct access of free Hb to the subendothelial space and VSMC. In ED a part of the atherosclerotic process also comprises endothelial injury, denudation, and ulceration, creating localized plaques and exposure of thrombogenic surfaces.

The combination of atherosclerotic endothelial dysfunction and HBOC represents an adverse synergy. Vasodilation is likely to be replaced by vasoconstriction in most vascular beds, accounting for the rise in the systemic and pulmonary arterial blood pressures. The adverse consequences of the action of free Hb are amplified in the presence of endothelial dysfunction. When ED and atherosclerotic changes affect the coronary circulation, endothelium-dependent protection of adequate blood supply may be compromised by the presence of fixed obstructions and dysfunctional vasoregulation.

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EFFECT OF INJECTION OF ARTIFICIAL RBCS ON MURINE HEMORRHAGIC HYPOTENSION MODEL

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Background: Availability of suitable blood for emergency transfusion is sometimes insufficient, so substitutes are needed. In the present study, we examined the effect of injection of rhodamine-labeled artificial RBCs (0.25 μm in diameter; Artif Cells, Blood Substitutes, Biotechnol. 35: 81, 2007) on murine hemorrhagic hypotension model.
Materials and Methods: Under isoflurane anesthesia, a cranial window was opened above the left parieto-temporal cortex of C57BL/6J mice (n = 18). Bilateral femoral arteries and tail vein were catheterized. An oxygen electrode (Bio Research Center) was placed near the branch of the left MCA with a reference Ag-AgCl electrode. Changes in brain microvasculature, blood pressure, partial pressure of oxygen in brain tissue (PO_2), and CBF measured by laser Doppler flowmetry were continuously recorded. Blood gases were regularly measured. FITC-labeled RBCs were injected into the circulating blood in all cases, and their movement through single capillaries in the ROI of brain parenchyma (50 μm depth) was monitored and recorded continuously with a video camera (30 frames/s; JCBFMetab. 25: 858 - 867, 2005). Hemorrhagic hypotension was induced by blood withdrawal (0.6 ml, 20 min) (n = 18), then the same amount of saline (n = 6) or aRBCs (n = 6) or whole blood (n = 6) was systemically injected and parameter values in the three groups were compared.
Results: Hemorrhagic hypotension to 31.8 ± 3.6% of the baseline blood pressure produced severe microvascular derangement with sluggish flow, disappearance of most FITC-labeled RBCs from capillaries, and development of RBC sludge and aggregates in arterioles and venules. Tissue PO_2 decreased to 70.0 ± 5.2% ($P < 0.05$) of the control value after hypotension.

- Saline injection group: BP, CBF, and tissue PO_2 showed slight but temporary improvement, then decreased gradually and all mice died within five hours.
- aRBCs injection group: Penetration of red 0.25 μm aRBCs through capillaries and transient resumption of capillary flow were observed. BP, CBF, and tissue PO_2 improved gradually. One hour after injection, tissue PO_2 had recovered almost to the baseline level. The survival rate was increased compared with the saline injection group.
- Whole blood injection group: High cerebral circulation and high tissue PO_2 (above baseline) were maintained. The survival rate was increased further, compared with the aRBCs group.

Conclusions: Administration of aRBCs to emergent ischemic tissue transiently improved the microcirculation. Supply of oxygen via improved capillary flow might temporarily rescue anoxic neurons. Use of aRBCs transiently improved the brain microcirculation in hemorrhagic hypotension, and might be therapeutically effective when immediate blood transfusion is not possible.

Poster selected for Tsuchida Prize Oral Presentation

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INFLUENCE OF OXYGEN AFFINITY OF PEGYLATED HEMOGLOBIN ON MICROVASCULAR PERFUSION DURING INDUCED ACUTE EXTREME HEMODILUTION

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Background: Design of high O_2 affinity has been proposed as a way to target O_2 release within the vascular network since high affinity favors off-loading O_2 to hypoxic areas and to overcome the vasoactivity autoregulatory mechanisms. Accordingly, the neutralization of vasoactivity with hexaPEGylated Hbs may be an additive or synergy of its high affinity and the induced super perfusion obtained by PEGylation. Low-affinity Hb preferentially releases O_2 to oxygenated portions of the microcirculation causing the additional O_2-carrying capacity to be ineffective for delivering O_2 to hypoxic tissues. Excess O_2 exposure to arterioles causes metabolic autoregulation and microvascular constriction reducing perfusion and O_2 delivery to the tissue. PEG-Hbs are a class of blood substitutes with excellent volume expansion properties, which have been shown to release O_2 to hypoxic tissue, and have documented maintenance of cardiovascular and microvascular function. The study objective was to investigate if the neutralization of vasoactivity using redesigned PEG-Hbs with low O_2 affinities still supports PEGylation-induced supra perfusion.
Material and Methods: Hamsters fitted with a dorsal window chamber that allows for microvessel assessments *in vivo* without complications of anesthesia were studied using video microscopy. PEG-Hb solutions (4% Hb) were introduced with an extreme exchange transfusion protocol, which resulted in a 75% reduction in systemic hematocrit and ~1.1% plasma Hb. P50 of the study solutions measured with a co-oximeter were: 32.0 mmHg (P50 –32, Thiocarbamoyl-P5K4 αα Hb) and 5.4 mmHg (P50–5, Propryl- P2K6 αα Hb.