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HEMORHEOLOGICAL IMPLICATIONS OF PERFLUOROCARBON BASED OXYGEN CARRIER INTERACTION WITH COLLOID PLASMA EXPANDERS AND BLOOD

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Abstract

Perfluorocarbon (PFC) emulsion based oxygen carriers lack colloid osmotic pressure (COP) and must be administered with colloid-based plasma expanders (PEs). Although PFC emulsions have been widely studied, there is limited information about PFC emulsion interaction with PEs and blood. Their interaction forms aggregates due to electrostatic and rheological phenomena, and change blood rheology and blood flow. This study analyzes the effects of the interaction between PFC emulsions with blood in the presence of clinically-used PEs. The rheological behavior of the mixtures was analyzed in parallel with *in vivo* analysis of blood flow in microvessels using intravital microscopy when administered in a clinically relevant scenario. The interaction between the PFC emulsion and PE with blood produced PFC droplets and red blood cell (RBCs) aggregation, and increased blood viscosity. The PFC droplets formed aggregates when mixed with PEs containing electrolytes, and the aggregation increased with the electrolyte concentration. Mixtures of PFC with PEs that produced PFC aggregates also induced RBCs aggregation when mixed with blood, increasing blood viscosity at low shear rates. The more viscous suspension at low shear rates produced a blunted blood flow velocity profile *in vivo* relative to non-aggregating mixtures of PFC and PEs. For the PEs evaluated, albumin produced minimal to undetectable aggregation. PFC and PEs interaction with blood can affect sections of the microcirculation with low shear rate (e.g. arterioles, venules, and pulmonary circulation) because aggregates could cause capillary occlusion, decrease perfusion, pulmonary emboli, or focal ischemia.

Keywords

Blood; viscosity; dextran; hydroxyethyl starch; human serum albumin; hydroxypropyl cellulose; hemodilution; blood substitutes; microcirculation

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1. INTRODUCTION

The development of a product suitable to replace blood volume and transport oxygen in cases of major blood losses has been the focus of many research groups worldwide for several years. The ideal product should maintain intravascular volume while carrying and delivering oxygen to tissue. Perfluorocarbon (PFC)-based oxygen carriers are synthetic blood substitutes.¹⁻² PFCs are oil compounds with high capacity to dissolve gases. Due to the hydrophobic nature of PFCs, they must be emulsified in order to be suitable for intravascular administration.² PFC emulsions have the capacity to dissolve large amounts of oxygen from low Van der Waals forces between PFC molecules and deliver oxygen based on concentration gradients.² PFC emulsions used as oxygen carriers should have adequate physicochemical properties such as stability and compatibility with physiological conditions. To achieve intravascular compatibility, the PFC emulsions must: i) not activate the immune response; ii) have physiological pH; iii) maintain constrained droplet size; and iv) exhibit stability. Limitations of immune function and complement activation had been reported before for PFC emulsions. The adverse reactions included flu-like symptoms and febrile responses in healthy volunteers.³ Droplet size is an essential determinant for PFC emulsion compatibility with the immune system,⁴ while phosphate buffers in the aqueous phase of the emulsions preserve the long term stability of the PFC emulsion.³⁻⁴

PFC emulsions with adequate physicochemical properties can be attained in laboratory production. However, in order to be used in the amounts required to transport a significant amount of oxygen, PFC emulsions need maximal compatibility with blood components and lack of immune reaction. Since PFC emulsions lack COP they must be mixed or co-injected with oncotic active fluids, generally colloid-based plasma expanders (PEs) to prevent fluid extravasations and edema.⁵ PEs interact physically and chemically with the PFC emulsion and blood when injected into the circulation.⁵ PEs mechanisms of interaction with PFC emulsion involve electrostatic and rheological phenomena.⁶ The interaction between PEs and PFC emulsion can cause undesirable outcomes such as promoting the formation of RBCs and PFC droplets aggregates. These aggregates could be weak and reversed by gentle shaking or could be strong, depending on the magnitude of the net attractive forces.⁷ The effect of aggregation *in vivo* is unknown; aggregates can probably break down under high shear rates, or occlude capillaries and limit tissue perfusion.⁸ The success of products intended to replace blood can be gauged by their ability to maintain adequate microcirculatory function, defined by the degree of perfused capillaries. In order to accomplish its function and unlike other pharmacological agents, PFC emulsions should be administered at doses as high as 4 g per kg body weight, which makes the previously described interaction issues more evident.^{2, 9}

In addition to having compatible biophysical properties such as oxygen carrying capacity, stability and adequate particle size, a clinically successful PFC emulsion needs to be fully compatible with blood and PEs. Previous studies have ignored the interaction between the PFC emulsion, PEs and blood. One exception is Nolte *et al*, who explored the compatibility of Oxygent™ (Alliance Pharmaceutical Corp., San Diego, CA), which is a PFC emulsion, with different colloid PEs on the microcirculation after acute normovolemic hemodilution.¹⁰ In a hamster model, Oxygent™ with 6% w/v 60 kDa dextran (Schiwa, Glandorf, Germany)

showed RBC aggregation and increased leukocyte activation and sticking to vascular endothelium in the microcirculation.¹⁰ The negative results observed with 60 kDa dextran were attributed to two hypotheses: i) aggregate formation between Oxygent™ and dextran and ii) coagulation and complement activation by the mixture.⁸ Unfortunately, the mechanism of interaction between Oxygent™ and dextran was not further studied, and Oxygent™ *in vitro* rheological and biochemical analysis did not include the potential interaction between the emulsion and RBC in the presence of PEs.¹¹ Interestingly, minor impairment of microvascular perfusion and limited inflammatory response to Oxygent™ was observed with other clinically used PEs such as 6% w/v hydroxyethyl starch (200 kDa; Schiwa, Glandorf, Germany), 3.5% w/v gelatin (30 kDa; Haemaccel™; Behring, Marburg, Germany), and 3.5% w/v albumin (67 kDa; Immuno, Heidelberg, Germany).¹⁰ Oxygent™ reached phase III clinical trials, but they were suspended in 2001 due to adverse outcome.¹² Similarly, Fluosol™ (Alpha Therapeutic Corp., Los Angeles, CA), a first-generation PFC emulsion and the only blood substitute approved to date by the U.S. Food and Drug Administration (FDA) for clinical use, was used in more than 40,000 subjects and then removed from the market due to stability issues.¹³ The large doses needed to achieve physiological relevance in terms of oxygen transport makes PFC based oxygen carriers a completely non traditional pharmacological agent, in addition to their potential interaction with PEs and blood components.⁹

This study was designed to analyze the rheological behavior of mixtures of colloid PEs and PFC emulsion with and without RBCs *in vitro*, and the implications of its rheological behavior in the microcirculation *in vivo*. We aim to establish the relation between physical suspension properties and microvascular changes induced by the infusion of colloid PEs and PFC emulsion. The colloidal PEs selected were: i) Human serum albumin 200 kDa (HSA) and ii) Hydroxyethyl starch 67 kDa (HES). HSA is a naturally occurring plasma protein that has long been considered as a gold-standard PE with limited immune reactions.¹⁴ HSA is derived from pooled human plasma serum, presenting risk of infectious disease transmission, although it is generally considered safe.¹⁴ HES is a widely used synthetic colloid derivative from amylopectin.¹⁵ HES has been clinically used to restore cardiovascular volume in patients with hemorrhagic shock.¹⁶ As positive control solutions to be mixed with the PFC emulsion and blood *in vitro*, we selected: i) Dextran 500 kDa (Dx) and ii) hydroxypropylcellulose 100 kDa (HPC). Dx is a polydisperse mixture of glucose polymers of various sizes and molecular weights. Dextran has been widely used to maintain circulatory dynamics during various types of shock and in the setting of ischemia-reperfusion injury.¹⁷ HPC is a cellulose ether derivative with high solubility in aqueous media that is used in small amounts as an intravascular drug carrier.¹⁸ Dx 500 kDa and HPC were used in the study as positive control solution because there is documented evidence of their RBC aggregation-inducing properties.¹⁹

2. MATERIALS AND METHODS

2.1 Perfluorocarbon emulsion

The PFC emulsion contains 58% weight/volume (%w/v) of perfluorooctyl bromide (CF₃(CF₂)₇Br; Exflur Research Corporation®, Round Rock, TX), soybean lecithin

Epikuron 170, (Cargill Company®, Hamburg, Germany) as an emulsifying surfactant, (+)- α -tocopherol (Sigma Aldrich®, St. Louis, MO) as antioxidant, Dextrose 50% for osmolality, and alginic acid sodium salt solution (Sigma Aldrich®, St. Louis, MO) as a viscosity agent. Briefly, PFC, soybean lecithin and the continuous phase were first homogenized (Heidolph SilentCrusher M®, Schwabach, Germany) and then emulsified (Microfluidizer M-110Y, Microfluidics Corp®, Newton, MA) until the mean particle size was below 250 nm. The PFC emulsion was heat sterilized at 100 °C for 40 minutes and then stored at 4 °C. The PFC emulsion properties are: i) Osmolality 252 ± 7 mOsm/kg H₂O; ii) Mean particle size: 234 ± 14 nm; and iii) pH: 7.34 ± 0.03

2.2 In vitro studies

2.2.1 Sample preparation

2.2.1.1 PFC emulsion interaction with colloid PE in presence or absence of RBCs: The mixtures of PFC and clinically-used PEs were analyzed in the presence and absence of RBCs. Whole blood samples were withdrawn from healthy Syrian golden Hamsters with a weight between 55 and 65 g into heparinized tubes. Blood samples were mixed with a suspension of 80% v/v PFC emulsion and 20% v/v PE or control solution to a final hematocrit (Hct) of 30%. The following colloid PEs were used for the mixtures in presence of RBCs: i) HES; ii) HSA; and as control solutions: i) Dx and ii) HPC. The same PEs and control solutions were used to study the interactions between PFC emulsion and PE in the absence of RBCs.

2.2.1.2 Effect of electrolyte concentration: In order to study the PFC emulsion interaction with different electrolyte concentrations in the plasma expander's solvent (saline solution as the aqueous phase) in the absence of RBCs, the hydroxyethyl starch (HES) dry powder was diluted in the following mediums: 1) deionized water; 2) 0.9% w/v NaCl (sodium chloride) and 3) Plasma-Lyte A (Baxter Healthcare Corporation®). Mixtures of 20% v/v HES solution and 80% v/v PFC emulsion were analyzed.

2.2.2 Rheological behavior—Rheological properties of the mixtures *in vitro* in the presence and absence of RBCs were determined with the hybrid rheometer (DHR) from TA Instruments® using a cone and plate geometry with a sample volume of 0.6 mL at a temperature of 37 °C; measurements were made in duplicate. The mixtures of PFC and different clinically-used PEs or control solutions in the presence and absence of RBCs were done the day of the experimentation and analyzed at steady-state. The shear rate was gradually increased from 10^0 to 10^3 s⁻¹ and then gradually decreased from 10^3 to 10^0 s⁻¹ to identify hysteresis.²⁰

PFC emulsion, PEs, control solutions and blood were individually studied to characterize fluid and flow properties. Viscosity (η) was measured as a function of shear rate ($\dot{\gamma}$), and their relationship was fitted using the power law constitutive model as given by equation 1,²¹ where “m” is the consistency index and “n” is a dimensionless quantity known as the flow index. The “n” value was obtained for each sample. A value of $n = 1$ corresponds to a Newtonian fluid while $n < 1$ corresponds to a shear thinning fluid. Lower n indicates a stronger shear thinning behaviour.^{20–22}

$$\eta = m\dot{\gamma}^{n-1} \quad (1)$$

Hysteresis was quantified as an area between the curves (hysteresis loop area) using the Cross model,^{20, 21, 22} as given by equation 2

$$\eta = \eta_{\infty} + \frac{\eta_0 - \eta_{\infty}}{1 + (m\dot{\gamma})^p} \quad (2)$$

where η_0 is the zero shear viscosity and η_{∞} is the infinite shear viscosity. The parameter “p” is a dimensionless index for the degree of dependence of viscosity on shear rate. The parameter “m” is the consistency index.

2.2.3 Aggregation Index and microscopic inspection—The aggregation index for the *in vitro* mixtures with RBC and without RBCs were measured using a photometric rheoscope (Myrenne Aggregometer®, Germany).²³ The aggregation index “M” was determined as follows: the sample was first exposed to a shear rate of 600 s⁻¹, the rotation was then stopped and the average change of light transmittance through a 0.35 mL sample of the mixture was recorded for 10 seconds. When no aggregation occurs the light transmission remains constant and the aggregation index is 0.²⁴ Additionally, microscopic evaluation of the *in vitro* mixtures with and without RBCs were completed by direct visualization under transmitted light (BX51WI; Olympus, New Hyde Park, NY) using a 20X objective. Images were then taken and analyzed offline to identify aggregates.

2.3 In vivo studies

To study the interaction of PEs and PFC emulsion with blood *in vivo* and the effect on blood flow, an acute isovolemic hemodilution was performed in 159 – 168 g anesthetized Sprague-Dawley rats. The microcirculation was studied using the rat’s cremaster muscle preparation combined with high-speed intravital video microscopy.

2.3.1. Animal preparation—All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (Institute For Laboratory Animal Research, National Research Council, National Academy Press, Washington, DC, 1996) and approved by the University of California, San Diego Animal Subjects Committee. Sprague-Dawley rats were anesthetized by intra peritoneal injection of 60 mg/kg of pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, IL); additional anesthesia was administered as needed during the experiment. The left femoral artery was catheterized for blood withdrawals and pressure measurements. The right jugular vein was also catheterized for anesthesia administration and PFC emulsion and PE mixture infusion. Catheters were flushed with heparinized saline (30 IU/ml) to prevent clotting. An animal’s temperature was maintained at 37 °C and a tracheal tube was implanted to support spontaneous ventilation. Then, rats were subjected to rat cremaster muscle preparation and exteriorization.²⁵ The exposed muscle was covered with polyvinylidene chloride (Dow Corning, Indianapolis, IN, USA) to preserve tissue moisture and prevent exposure to air. Animals were euthanized at the end of the experiment.

2.3.2. Acute isovolemic hemodilution protocol—An isovolemic hemodilution state was induced by lowering the Hct to 30% with the mixture of 80% v/v PFC emulsion and 20% v/v PEs. The volume of the exchange transfusion used to achieve 30% Hct was estimated as $0.024 \times V \times \Delta \text{Hct}$, where V is the animal blood volume in milliliters (estimated as 6% of the rat's weight in grams), and ΔHct is the difference between the initial and desired Hct value. Mixtures were infused through the jugular vein, while an identical volume of blood was withdrawn from the femoral artery. The exchange transfusion rate was set at 0.3 ml/min to avoid rapid shifts in systemic values and water-volume redistributions. Baseline systemic, blood chemistry and microhemodynamic measurements were completed at baseline (before the exchange protocol) and after the moderate hemodilution. The animals were given a 5 min recovery period after the hemodilution before data acquisition, which continually followed for over 30 minutes. The experimental protocol is schematically shown in Figure 1.

2.3.3. Experimental setup for high speed video microscopy—Video images were obtained with a high-speed video camera (Fastcam 1024 PCI, Photron USA) with a 1 megapixel chip. An intravital microscope (BX51WI; Olympus, New Hyde Park, NY, USA) was used with an Olympus 40 $\times$ water-immersion objective (NA = 0.7) and a long working distance condenser (Instec, Boulder, CO, USA). An additional lens was placed before the camera to produce an effective pixel width of 0.3 μm relative to the object plane. A blue filter with peak transmission at 400 nm (Spectra Physics, no. 59820) was placed before the light source to maximize light absorption by the RBCs. The microscope was focused on the diametrical plane of the arteriole and the three videos were recorded at a rate of 3000 fps (frames per second). Images were recorded on a hard drive for offline analysis.²⁶

2.3.4. Arteriolar blood flow velocity profiles—Velocity profiles were calculated using cross correlation in order to calculate the instantaneous blood flow velocity at different points along the radial direction of the micro-vessel.²⁷ Automatic analysis of the videos was completed using a custom Java-based macro developed for ImageJ (<http://rsb.info.nih.gov/ij/>). This macro calculates image velocimetry by capturing the flow velocity instantaneous flow fields in the images. Briefly, an image for a frame and its consecutive acquisitions are segmented into sub-images. Cross correlation coefficients are calculated as a function of a longitudinal shift applied between sub-images. Relative displacement is calculated as the difference in the position where maximum correlation coefficients are found. After computing the longitudinal displacements for the various sub-images at the same radial position they are averaged. Velocity is calculated as the average longitudinal shift displacement divided by the time between frames (0.5 ms). The previously described experimental setup allows for velocity measurements between 0.3 and 250 mm/s. The cross correlation process is repeated for equally distributed radial positions and the velocity profile is obtained for each two consecutive frames. Mean velocity is calculated as the mathematical average of the velocity profile. The velocity profiles were fitted using equation 3, where $V(r)$ is the normalized velocity at radial position r and R is the radius of the vessel. The equation is dimensionless.

$$V(r)=1 - \left(\frac{r}{R}\right)^K \quad (3)$$

The shape of the velocity profiles and their deviation from a parabola is indicated by an augmentation or decrease of the value of K. ^{26, 28} As the value of K increases, the velocity profile becomes more blunted in shape, which corresponds to an increase in viscosity at lower shear rates. ²⁸ This analysis of the velocity profiles let us correlate the changes in blood flow in microvessels in the microcirculation to the *in vitro* rheological behavior of the PEs and PFC emulsion interaction in the presence of RBCs. The flow velocity profiles shape has been reported to be dependent on blood aggregation. ²⁹

2.3.5 Wall Shear stress—The wall shear stress (WSS) was calculated to determine blood flow and endothelial cells mechano-transduction. WSS depends on blood viscosity and wall shear rate (WSR). WSR was calculated as the rate of change in velocity near the wall, estimated as the change in velocity ($\frac{dv}{dx}$) relative to the distance to the wall ($\frac{v}{x}$). WSS was calculated as the product of viscosity (at specific WSR) and WSR, $WSS = \eta \times WSR$.

2.3.6 Rheological behavior and aggregation—Whole blood samples were withdrawn from the animals into heparinized tubes at baseline and 30 minutes after hemodilution. The rheological behavior of the blood samples was immediately analyzed using the hybrid rheometer (DHR) from TA Instruments® using cone and plate, as described for the *in vitro* experiments. The aggregation index was also measured as described before.

2.4 Data analysis

Data is presented as mean $\pm$ standard deviation (SD). All statistical analysis and data fitting were completed using Prism 5.0 (Graphpad Software, Inc., San Diego, CA). Data between groups was analyzed using Non parametric analysis of variance (ANOVA, Kruskal-Wallis test). When appropriate, post hoc analyses were performed with the Tukey multiple comparison test. Changes were considered statistically significant if $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 In vitro studies

3.1.1 Mixtures in the presence of RBCs

3.1.1.1 Rheological behavior: When studied as separate components between the shear rates of 10^0 to 10^3 s^{-1} , the rheological measurements for PFC emulsion, PEs, control solutions and blood showed a shear thinning behavior with a flow index value below 1. The results are summarized in Table 1. Results for the rheological behavior of the mixtures of PFC emulsion, PEs, and blood are shown in Figure 2.

For the mixtures of PFC emulsion and RBCs with HES, Dx or HPC the results are presented in Figures A, C and D, respectively. The ascending and descending flow curves show hysteresis, which is usually referred to as a ‘Thixotropic loop’. Hysteresis can be interpreted as changes in the structure of the system because of the presence of aggregates. These

aggregates would change as shear forces are applied. The structures break down during the upward part of the test (from 10^0 to 10^3 s^{-1}) and viscosity decreases as shear rate is decreased in the downward part of the test (from 10^3 to 10^0 s^{-1}). Figure B shows no hysteresis, probably because no aggregates were formed in the mixtures of PFC emulsion and RBCs with HSA. To quantify the hysteresis, the loop area was calculated for each mixture, and the results are summarized in Table 2.

The viscosity of blood at 30% Hct increased by 237% when the mixture of PFC emulsion and HPC was added. Adding the mixture of PFC emulsion and Dx to blood increased the viscosity by 144%. The mixture of PFC emulsion and HES increased blood viscosity by 50% and the mixture of PFC emulsion and HSA increased blood viscosity by only 9%. The largest increase in blood viscosity correlates with the largest hysteresis loop area. Therefore, the viscosity and the hysteresis loop area may be related to structures formed by the interaction of PFC emulsion and PEs or control solutions with blood, since Hct was identical, suggesting that aggregates are responsible of the increase in viscosity.

Macro proteins like fibrinogen and globulins increase blood viscosity by interacting with RBCs and producing RBC aggregation.³⁰ Fibrinogen induced RBC aggregation is a very specific process due to RBC-to-RBC binding, where as PEs induced RBC aggregation includes multiple factors.³¹ The mechanism for dextran-induced RBC aggregation is dependent on molecular weight.¹⁹ Although, dextrans ranging in molecular weights between 40 and 2000 kDa produced maximal aggregation at 500 kDa.¹⁹ Therefore, Dx alone may be responsible for the RBCs aggregation and confirms the importance of molecular mass in the formation of aggregates.

Strongly aggregated systems usually show much less hysteresis than weakly aggregated systems; hence, it is not valid to assume lack of aggregation for the HSA and PFC emulsion mixture with RBCs by simply using the rheological data. The presence of aggregates was validated with microscopic observations. The RBC aggregation is important because it is largely responsible for the profound shear thinning behavior of blood (e.g., decreasing blood viscosity with increasing shear rate due to the dispersion of the aggregates). This also has substantial effects on *in vivo* hemodynamics and can adversely affect RBC distribution and flow dynamics in the microcirculation.

3.1.1.2 Aggregation Index and microscopic inspection: The results for the aggregation index using the Myrenne aggregometer are summarized in Figure 3 for the mixtures of PFC emulsion and PE or control solution in presence of RBCs. The largest aggregation was observed with the HPC, followed by Dx and the HES. This result is consistent with the reported hysteresis loops. Micro-photographies for the mixtures are presented in Figure 4. RBCs aggregation induced by hydroxypropyl cellulose and dextran of high molecular weight is well known and has been previously reported.⁸ Using their images as a reference (bottom pictures) it is possible to interpret the effects of HES and HSA (top pictures) on the PFC emulsion and RBCs. The mixture with HES induced the aggregation of PFC droplets whereas the mixture with HSA does not present aggregates of any kind (RBCs or PFC droplets). Previous studies have shown that HSA surface charge decreases RBC aggregation by macromolecules by increasing the repulsion forces as sialic acids on the surface of the

cells gives then a negative zeta potential.^{8, 32} Thus, HSA enhances surface charge and electrostatic repulsive forces thus inhibiting RBC and PFC interaction.^{8, 32}

3.1.2 Mixtures in the absence of RBCs—In order to study the interaction of the PFC emulsion alone with the PE or control solution, we studied mixtures in the absence of RBCs.

3.1.2.1 Rheological behavior: The rheological properties of the mixture PFC emulsion and PE or control solution were measured, and shear rate vs. viscosity plots were obtained. Data was analyzed similarly to the mixtures in the presence of RBCs in terms of hysteresis loop area. The results are summarized in Table 3. Similarly as when RBCs were present, the mixtures of PFC emulsion with HES, Dx and HPC presented hysteresis. It seems that in presence of HES, Dx or HPC the PFC droplets form aggregates. These aggregates were broken down as the shear rate increased, rearranging the structure of the aggregates. On the other hand, the mixture of the PFC emulsion with HSA did not present hysteresis, and the solution viscosity value at 100 s^{-1} (Table 3) was the lowest as compared to HES, Dx and HPC.

3.1.2.2 Aggregation Index and microscopic inspection: The mixtures in absence of RBCs were analyzed in the aggregometer. The aggregation indexes are summarized in Figure 5. The aggregation index shows the presence of aggregates of PFC droplets between the mixtures: PFC emulsion-Dx showing the higher followed by PFC emulsion-HPC, and PFC emulsion-HES. The PFC emulsion mixture with HSA did not produce any aggregation of PFC droplets.

The mixtures were observed under the microscope and the results are presented in Figure 6. The aggregation of the PFC droplets is evident for the mixtures of PFC emulsion and Dx, HPC, or HES. There is strong agreement between the aggregation index, rheological behavior, and micro-photography results. The aggregate formation between PFC droplets with Dx confirms the incompatibility of PFC emulsion and dextrans previously reported.¹⁰

There is clear evidence of the aggregation of PFC droplets by HES, Dx and HPC. On the other hand, HSA did not affect the structure of the PFC emulsion. It is important to note that HES, Dx and HPC have a relatively higher sodium electrolyte concentration of 154 mEq/L than HSA (72.5 mEq/L), which likely plays a role in the tendency for the PFC droplets to aggregate.⁴ The presence of an adsorbed anionic surfactant (soy bean lecithin) over the emulsion droplets creates a surface charge and a double layer as a result of the presence of an unequal distribution of counter ions and co-ions. The magnitude of the repulsive energy between the PFC droplets determines their tendency to form aggregate units or to remain as individual particles. This energy decreases with separation distance, but increases with electrolyte concentration and electrolyte valency.⁴

3.1.3 PFC emulsion interaction with the PEs solvent—Interaction between PFC emulsion and PE's solvent (saline solution as the aqueous phase) at different electrolyte concentration in absence of RBCs was analyzed with mixtures 20% v/v HES solution and 80% v/v PFC emulsion.

3.1.3.1 Aggregation Index and rheological behavior: The aggregation index and the rheological properties as a function of shear rate for the mixtures of 80% v/v PFC emulsion and 20% v/v HES solutions were measured. The results are summarized in Table 4 and Figure 7. PFC emulsion mixed with HES diluted in deionized water presents zero hysteresis area, low viscosity and low aggregation index, whereas with a sodium electrolyte concentration of 140 mEq/L and 154 mEq/L the hysteresis loop area, the viscosity and the aggregation index increased proportionally with the electrolyte concentration.

3.1.3.2 Microscopic inspection: The mixtures of PFC emulsion and HES solutions were observed under the microscope; images are shown in Figure 8. The PFC droplets presented aggregation when suspended in NaCl or plasma-lyte. The microphotographs and aggregation index are in agreement with the rheological results. Therefore, sodium electrolyte concentration appears to affect PFC emulsion stability and structure.

3.2 In vivo studies

Based on the *in vitro* results, we selected two solutions to be tested *in vivo*: (i) an aggregating solution with sodium electrolyte concentration equal to 154 mEq/L (HES) and (ii) a non-aggregating solution with sodium electrolyte concentration of 72.5 mEq/L (HSA). A total of four rats were studied divided into 2 groups, namely: *High-Electrolyte* (154 mEq/L), and *Low-Electrolyte* (72.5 mEq/L); the animals were hemodiluted with 80% v/v PFC emulsion mixed with 20% v/v HSA or HES.

3.2.1 Systemic and blood gas parameters—Systemic parameters for each resuscitation solution are presented in Table 5. Fluorocrit was calculated as the ratio of PFC to whole blood in centrifuged samples in capillary glass tubes. There was no statistically significant difference for any of the parameters between the two solutions.

3.2.2 Blood Rheology—Table 6 shows a comparison of the rheological properties of rat blood at normal Hct of 49%, with a 30% Hct and between the animals treated with the two different solutions. Comparison is presented in terms of viscosity at 100 s^{-1} and hysteresis loop area. The rheological behavior results of the *in vivo* measurement are in agreement with the *in vitro* results presented before. The HES presented a hysteresis loop while HSA did not. This result is an indication that observations *in vitro* could be extrapolated to understand the *in vivo* behavior of different PEs and artificial oxygen carriers.

3.2.3 Arteriolar blood flow velocity profiles—Velocity profiles were built for the flow in the arterioles of the rat's cremaster muscle at baseline conditions and 30 minutes after hemodilution to quantify the changes induced by the presence of the solutions over the blood flow dynamics and the fluid-vessel wall interaction. The parameter K increased after hemodilution with HES, but not for the hemodilution with HSA. The velocity profiles are shown in Figure 9. An increase in the parameter K is related to profiles with a more blunted shape as has been reported by Bishop *et al.*²⁹ Aggregates present in the blood-stream need higher shear rates to be broken apart than non-aggregated blood. Aggregates near to the center of the vessel remain unaffected by flow since the shear rates are near zero and thus they move as a unit resulting in the blunted velocity profile. Changes in the velocity profile

are shear rate-dependent and a more blunted shape is related to an increase in viscosity at low WSR and a subsequent increase in flow energy expenditure.

Blood flow through the cardiovascular system is driven by pressure that is higher during systole than diastole. This cyclic change produces periods of higher shear rates which can breakup small aggregates.^{7, 30} However during diastole, WSRs may be too low and will have no influence on the aggregates. This is seen in the values of the parameter K, where they are always smaller for the velocity profiles during systole as compared to those during diastole, which was true for both the high and low electrolyte concentrations. This result is consistent with the results obtained in the *in vitro* rheological characterization and with those previously reported by Chien *et al.*³⁰ The K values for the high electrolyte group were higher compared to the K value for baseline. In contrast, the values of K decreased after hemodilution with low electrolyte solution compared to the baseline. Changes in WSS are consistent with the changes in K of the velocity profiles. For the high electrolyte concentration mixture the WSS was only decreased by 10% of the baseline value while for low electrolyte concentration mixture the decrease was near 50% of the baseline value as shown in Figure 10.

The mixtures of PFC and HES with blood promoted the formation of PFC droplets aggregates and affected blood flow in the microcirculation in a shear rate-dependent way. The presence of aggregates in the microcirculation induced changes in the shape of the velocity profile and affected the interaction of the flowing blood with the endothelial cells, increasing WSS and endothelial mechano-transduction.³³ The impact of RBCs aggregation on an entire vascular bed is intrinsically difficult to describe because RBCs aggregation might exert opposite effects at different levels of the vascular network. As the microcirculation is an arrangement of narrow tubes, RBCs aggregation may promote accumulation in the center of the tubes, blunting of velocity profiles, increasing shear stress, and promoting the formation of cell-depleted layer of plasma. Thus, these changes can increase endothelial mechano-transduction and vasodilation, which reduces frictional resistance and therefore the apparent viscosity. On the other hand, because energy is required by rouleau disaggregation, and since capillary plugging in the terminal part of the vascular network or compaction stasis can be increased by RBCs aggregation, it may also tend to increase resistance to flow. Therefore, as the shear rate conditions of the circulation system are low (e.g. venous and pulmonary system),³⁴ aggregates could persist and cause occlusion of capillaries, pulmonary emboli, or strokes.⁸

The occlusion of capillaries due to the presence of aggregates was evident in our intravital microscopy study. The recorded videos show how the blood flow is transitorily blocked and subsequently restored as pressure builds upstream. On the other hand, increased WSS stimulates the production of vasodilators (e.g. nitric oxide, NO).³³ However, depending on the degree of the aggregation as in the case of PFC- and PE-induced RBC aggregation, the beneficial effects of increasing WSS may be limited by the presence of large aggregates (> 5 μm) and the energy needed to break up such aggregates. In regard to pulmonary circulation previous studies with PFC have reported that the disaggregation effect appears to limit the benefits of increased mechano-transduction as RBC aggregation was manifested as pulmonary hypertension and hypoxic events.^{35–36}

The factors involved in RBC aggregation need to be investigated in detail in the future. One of the most important areas where detailed information is insufficient relates to the mechanisms responsible of cell-to-cell and cell-to-PFC emulsion interaction. There are two current hypotheses on RBC aggregation: (i) the cross-bridging hypothesis and the (ii) depletion layer hypothesis.³⁷ The cross-bridging model is proposed to occur due to large macromolecules such as plasma proteins cross-linking binding sites on adjacent cells.³⁷ In contrast, the depletion model proposes that RBC aggregation occurs because of low local protein or polymer concentration near the cell surface leading to osmotic gradient, depletion of interaction, and attractive forces.³⁷ The sensitivity of RBC aggregation to PFC and PEs mixtures and electrolyte concentration may include both models: cross-bridging and osmotic gradients by PEs and electrolytes. We speculate that PFC emulsion-PEs-RBC interaction arises from the terminal portions of the polymers that are adsorbed onto the surfaces of adjacent RBCs and PFC droplets resulting in a cell-to-cell and cell-to-PFC emulsion aggregation in a process mediated by cations, which reduces repulsion forces by direct binding to the phospholipid head groups. However, more specific studies need to be completed to provide more conclusive evidence for the mechanism that leads to interaction between PFC emulsion and PEs with RBCs.

4. CONCLUSIONS

The main finding of this study is the evidence that electrolyte concentration and molecular mass of plasma expanders negatively affect the stability of PFC emulsions. There is a relation between the concentrations of sodium electrolyte in the different plasma expanders with the formation of aggregates in the PFC emulsion. Mixtures of PFC emulsion and Dx and HPC with blood caused RBC aggregation, whereas mixtures of PFC emulsion and HES with blood caused less RBC aggregation, and the mixture of PFC emulsion and HAS with blood did not produce any RBC aggregation. The formation of aggregates induced by the interaction of PE, RBCs and PFC emulsion negatively affected the viscosity of blood. The *in vivo* effect of the presence of aggregates was evidenced by changes in the shape of the velocity profiles in the microcirculation. Changes in the interaction between blood flow and microvascular endothelium was characterized by an increase in the wall shear rate and the wall shear stress for the high electrolyte solutions as compared to the low electrolyte solutions. An increase of the wall shear stress is related to a higher endothelial mechano-transduction, which could be related to changes in blood vessel tone.

In a clinical setting, the formation of aggregates in the PFC emulsion and between RBCs is an undesired outcome because it could cause systemic and pulmonary capillary collapse, pulmonary emboli or strokes, which result in poor tissue perfusion. The scenario where the PFC emulsions are meant to be used includes severe anemic stages and hypovolemia. As a result, the main goals of their intravascular use should be the restoration of the intravascular volume and maintenance of an adequate microvascular and tissue perfusion. The presence of aggregates within circulation not only impedes the fulfillment of these objectives, but it also can worsen the situation. The PFC emulsions need to be co-injected with a PE of low molecular mass and adequate electrolyte concentration in order to function appropriately in the human body and to produce the desired clinical outcomes without causing adverse effects. Based on the results presented in this study, a possible PE to be used in conjunction

with PFC emulsion may be HSA with balanced osmolarity. However, more research in this area is necessary to optimize the formulation of PEs in terms of the colloids and electrolytes used in conjunction with a PFC emulsion.

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LIST OF ABBREVIATIONS

*Dextran T500 MW 500 KDa, Pharmacia Fine Chemicals®, Uppsala, Sweden

**Cellulose MW 100 KDa, Hercules®, Aqualon Inc., Wilmington, DE

***Pentaspán, MW 200 KDa, degree of substitution of 0.6, B Braun®, Irvine, CA

****Flexbumin 25%, MW 67 KDa, Baxter®, Westlake Village, CA

COP	Colloid osmotic pressure
Dx*	Dextran (6% w/v)
HPC**	Hydroxypropyl cellulose (2% w/v)
Hct	Hematocrit
HES***	Hydroxyethyl Starch (10% w/v)
HAS****	Human serum albumin (2.9% w/v)
PE	Plasma expander
PFC	Perfluorocarbon
RBCs	Red blood cells
WSS	Wall shear stress
WSR	Wall shear rate

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Figure 1. Diagram of moderate normovolemic hemodilution

Baseline characterization was performed before hemodilution. Blood volume was exchanged with a solution of 80% v/v PFC emulsion and 20% v/v colloid PEs. RBCs: Red blood cells; Hct: hematocrit; Fct: Fluorocrit; BL: Baseline; HD: hemodilution; RE: resuscitation; PFC: Perfluorocarbon; PEs: Plasma expanders. Data was recorded at baseline and 30 minutes after the hemodilution. Figure taken and modified from²⁴.

A. Blood + PFC + HES (at 30% Hct) **B. Blood + PFC + HSA (at 30% Hct)** **C. Blood + PFC + Dx (at 30% Hct)** **D. Blood + PFC + HPC (at 30% Hct)**

Figure 2. Flow sweep at steady state from a shear rate of 10^0 to 10^3 s^{-1} (up) and 10^3 to 10^0 (down) for the mixtures PFC emulsion and PE or control solution in presence of RBCs (A) Blood-PFC emulsion and hydroxyethyl starch. (B) Blood-PFC emulsion and human serum albumin. (C) Blood-PFC emulsion and dextran. (D) Blood-PFC emulsion and hydroxypropylcellulose. PFC: Perfluorocarbon; PEs: Plasma expanders.

Figure 3. Myrenne (M) aggregation index of the mixtures PFC emulsion and PE or control solution in presence of RBCs

PFC: Perfluorocarbon; PE: Plasma expander; RBCs: Red blood cells.

A. Blood + PFC + HES (at 30% Hct)

B. Blood + PFC + HSA (at 30% Hct)

C. Blood + PFC + Dx (at 30% Hct)

D. Blood + PFC + HPC (at 30% Hct)

Figure 4. Pictures of the mixtures PFC emulsion and PE or control solution in presence of RBCs
Micrographs were contrast enhanced using histogram normalization. PFC: Perfluorocarbon;
PEs: Plasma expanders; RBCs: Red blood cells.

Figure 5. Myrenne (M) aggregation index of the mixtures between PFC emulsion and PE or control solution

PFC: Perfluorocarbon; PE: Plasma expander.

Figure 6. Mixtures between PFC emulsion and PE or control solution

Micrographs were contrast enhanced using histogram normalization. PFC: Perfluorocarbon; PEs: Plasma expanders.

Figure 7. Myrenne (M) aggregation index and hysteresis loop area of the mixtures between PFC emulsion and HES solutions as a function of PE solvent electrolyte concentration
PFC: Perfluorocarbon; PE: Plasma expander; HES: hydroxyethyl starch.

A. PFC + 10% w/v HES in NaCl

B. PFC + 10% w/v HES in plasma-lye®

C. PFC + 10% w/v HES in dionized water

Figure 8. Mixtures between PFC emulsion and HES solutions

PFC: Perfluorocarbon; HES: hydroxyethyl starch. The micrographs were contrast enhanced using histogram normalization.

Figure 9. Sample set of velocity profiles determined for systole and diastole (high and low maximum velocity), before and 30 minutes after a hemodilution

A. Human serum albumin with a low electrolyte concentration 72.5 mEq/L of sodium solution and B. Hydroxyethyl starch with a high electrolyte concentration solution 154 mEq/L of sodium. K value is normalized.

Figure 10. Wall shear stress changes at 30 minutes after a moderate hemodilution with high and low electrolyte concentration solutions

Table 1
Power law indexes for the PFC emulsion, PEs, control solutions and blood

Blood was diluted to 30% Hct with PBS (phosphate buffered saline). PFC: Perfluorocarbon emulsion; PEs: Plasma expanders.

Sample	n	Viscosity at 100 s ⁻¹ (cP)
Blood (at 30% Hct)	0.89	2.3 ± 0.3
PFC (alone)	0.99	2.8 ± 0.0
PFC + 10% w/v HES	0.69	8.0 ± 0.7
PFC + 2.9% v/v HSA	0.80	2.3 ± 0.0
PFC + 6% w/v Dx	0.98	5.8 ± 0.2
PFC + 2% w/v HPC	0.88	13.1 ± 0.5

Table 2
Viscosity and hysteresis loop area for the mixtures PFC emulsion and PE or control solution in presence of RBCs

Blood was diluted to 30% Hct with PBS; PFC:Perfluorocarbon emulsion; PEs:Plasma expanders.

Sample (at 30% Hct)	Viscosity at 100 s^{-1} (cP)	Hysteresis loop area (cP/s)
Blood	2.3 ± 0.3	none
Blood + PFC + HES	3.5 ± 0.4	0.6 ± 0.3
Blood + PFC + HSA	2.4 ± 0.4	none
Blood + PFC + Dx	5.6 ± 0.4	1.1 ± 0.8
Blood + PFC + HPC	7.8 ± 0.5	1.2 ± 0.7

Table 3
Viscosity and hysteresis loop area for the mixtures between the PFC emulsion and the PE or control solution in absence of RBCs

PFC: Perfluorocarbon emulsion; PEs: Plasma expanders; RBCs: Red blood cells.

Sample	Viscosity at 100 s^{-1} (cP)	Hysteresis loop area (cP/s)
PFC + HES	3.5 ± 0.4	1.4 ± 0.1
PFC + HSA	2.2 ± 0.0	none
PFC + Dx	5.1 ± 0.9	2.2 ± 0.3
PFC + HPC	8.3 ± 0.8	1.8 ± 0.2

Table 4
Viscosity and hysteresis loop area for the mixtures between the PFC emulsion and the HES solutions in absence of RBCs

PFC: Perfluorocarbon emulsion; PEs: Plasma expanders; RBCs: Red blood cells; HES: hydroxyethyl starch.

Sample	Viscosity at 100 s^{-1} (cP)	Hysteresis loop area (cP/s)
PFC + 10% w/v HES in NaCl	4.6 ± 0.6	2.3 ± 0.0
PFC + 10% w/v HES in plasma-lyte®	3.8 ± 0.2	1.3 ± 0.2
PFC + 10% w/v HES in dionized water	2.7 ± 0.9	none

Table 5
Macrohemodynamic parameters during shock model. Mean value $\pm$ standard deviation

PFC: Perfluorocarbon emulsion; Hct: hematocrit; Hb: hemoglobin; Fct: Fluorocrit.

Resuscitation Solution	Parameter	Baseline	Resuscitation 30 minutes
80% v/v PFC + 20% v/v HES	Hb, g/dl	14.4 $\pm$ 2.5	11.5 $\pm$ 0.8
	Hct, %	44.5 $\pm$ 4.9	31.5 $\pm$ 0.7
	Fct, %		7.0 $\pm$ 0.1
80% v/v PFC + 20% v/v HSA	Hb, g/dl	16.7 $\pm$ 0.6	10.8 $\pm$ 0.2
	Hct, %	49.0 $\pm$ 2.8	30.0 $\pm$ 0.1
	Fct, %		9.0 $\pm$ 2.8

Table 6
Viscosity and hysteresis loop area for the rat's blood with the two groups of solutions

Blood viscosity and hysteresis loop were measured from the blood collected at the end of the experiments. Additional samples non-diluted rat's blood and blood diluted to 30% Hct with PBS are presented as references. PFC: Perfluorocarbon emulsion.

Sample	Viscosity at 100 s^{-1} (cP)	Hysteresis loop area (cP/s)
Blood (at 49% Hct)	5.6 ± 0.7	none
Blood (at 30% Hct)	2.8 ± 0.1	none
Blood + PFC + HES	3.4 ± 0.4	0.4 ± 0.1
Blood + PFC + HSA	2.7 ± 0.1	none