

Comparison of the Macro-circulatory Effects of the New Generation Plasma Expander EAF-HexaPEGylated Albumin (EAF-PEG-BSA) in a Rabbit Hemorrhagic Shock Model: a Comparison with Conventional Crystalloid and Colloid Solutions

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Abstract

Introduction: HexaPEGylated albumin (PEG-BSA) is a new class of low viscosity Nitric Oxide (NO) producing active plasma expander. The macro-circulatory response to PEG-BSA of a 4 gm solution of PEG-BSA was studied in a rabbit hemorrhagic shock and resuscitation model and compared that of 0.9% sodium chloride and albumin 25%.

Methods: Five New Zealand white rabbits were anesthetized utilizing ketamine with diazepam and propofol. Endotracheal intubation was performed, animals were ventilated, and anesthesia maintained with isoflurane and diazepam. Following placement of vascular lines for measuring physiological variables, 45 minutes were allowed for equilibration prior to collecting baseline values laboratory and physiologic values. Animals were bled over 30 min to reach a Mean Arterial Pressure (MAP) of 30-41 mmHg. This pressure was maintained for an additional 30 min. Post-Hemorrhage (PH) values were recorded one hour after initiation.

Results: The hematocrit decreased by hemorrhagic shock, and the Heart Rate (HR) returned to the baseline in all 25% PEG-BSA rabbits and in 75% PEG-BSA, HR was greater than baseline. In the saline group, MAP and SvO₂ returned to baseline values, the restitution of the baseline value was least effective with albumin, while with PEG albumin the values returned to near control values. Cardiac Output (CO) did not return to the baseline values when resuscitated with saline or 25% albumin, but returned close to the baseline with PEG-BSA both with the 25% and exceeding the baseline value in the 75% group. The pH returned to baseline in all rabbits, whereas lactate increased, remaining elevated relative to the baseline.

Conclusion: The responses to the resuscitation by the three fluids in rabbit hemorrhagic chock models are distinct. Resuscitation with 0.9% sodium chloride, improves only MAP and SvO₂ suggesting improved responses. The PEG-BSA as well as albumin, improved MAP, SvO₂, HR, pH, and lactate, but PEG-BSA at lower levels compared to albumin.

Introduction

Hemorrhagic shock is a leading cause of morbidity and mortality throughout the world, with young healthy victims being the largest casualty, with some estimates upward of 5 million deaths each year. Current resuscitation strategies using crystalloid are limited, with lack of readily available, modestly priced alternatives that can be placed in ambulances or rescue helicopters both for civilian and military trauma that have been validated and verified to improve both short term and long term outcomes.

PEGylated proteins have emerged as excellent protein therapeutics in view of the increased half-life of these as compared to the parent protein of therapeutic interest [1]. PEGylation induced increase resistance of protein to degradation by proteases and masking of the protein core from the immune system *in vivo* have been the primary molecular properties of PEGylated protein therapeutics that has made PEGylation as a novel platform for to develop new protein therapeutics.

The development of PEG-Hb as potential oxygen therapeutics represents a major paradigm shift in the design of blood substitutes. The non-hypertensive nature of PEG H *in vivo* is considered to be a direct consequence of the PEGylation induced plasma expander-like properties of PEG-Hb. The PEGylation induced plasma expander like properties include the unusually high hydrodynamic volume (relative

to the mass of PEG-Hb adducts), high COP and high viscosity. PEG albumin also exhibits the plasma expander like solution properties. PEG-albumin, besides increasing the functional capillary density also induces vasodilatation thereby inducing supra perfusion. The suprapfusion activity of PEG albumin is achieved by enhancing shear-thinning of the RBC in the presence of PEG Albumin PEG-Hb and PEG albumin represent a new class of low viscosity plasma expanders are endowed with the unusual propensity to increase the endothelial NO production and inducing suprapfusion that is

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generally associated with high viscosity plasma expanders like dextran 500. Both PEG Albumin and PEG Hb have been advanced as potential oxygen carrying and non-oxygen carrying plasma expanders.

PEG-albumin, particularly the hexaPEGylated PEG-albumin [EAF (SP-PEG5K)-Albumin], generated by Extension Arm Facilitated PEGylation platform is one of the PEG albumins that is currently being tested as unique resuscitation fluids. Both PEGylation patterns and the optimum concentrations of PEG-albumin and the protocols ideally suited to elicit the best response when used as the resuscitation fluid has been the subject of considerable interest in recent years. Recent studies of PEG-albumins have revealed considerable support for their benefit at the microvascular level when used as the resuscitation fluid. Cabrales et al. compared extreme hemodilution with PEG hemoglobin and PEG-albumin [2]. This study showed that PEG-albumin solutions “maintained microvascular conditions with lower concentrations than conventional plasma expanders” [2]. Surprisingly, in addition, oxygen delivery by PEG-albumin was slightly than the delivery by PEG-hemoglobin under identical conditions [2]. The superiority of PEG-albumin relative to albumin solutions, and carbohydrate based plasma expanders has also been established.

In the current pilot analysis, a rabbit hemorrhagic shock and resuscitation model has been used to evaluate macro vascular physiologic responses function and the results have been compared to crystalloids as well as colloids (albumin).

Methods

After UC Davis IACUC approval, 5 mixed gender New Zealand white rabbits were studied. Animals were sedated with ketamine 50 mg/kg IM and then a 22-gauge catheter placed in the ear vein. Subsequently, anesthesia was induced with diazepam 0.5 mg/kg IV and propofol 2 mg/kg IV and animals endotracheally intubated using a 3.5-4.0 mm ID cuffed tube and then attached to a rebreathing circuit. Anesthesia was maintained with 1.5% isoflurane and a constant rate infusion of diazepam (12.5 µg.kg⁻¹.min⁻¹). The FiO₂ was kept at 0.21-0.25. Animals were mechanically ventilated using a tidal volume of 10 ml/kg and a rate sufficient to maintain an end-tidal partial pressure of CO₂ of 40 mmHg. Animals were instrumented with a femoral or ear arterial catheter placed for measuring Mean Arterial Blood Pressure (MAP), Pulse Rate (PR) and collecting arterial blood and a femoral/iliac venous catheter inserted into the vein for measuring Central Venous Pressure (CVP) and collecting mixed venous blood. A 45-min period was allowed for equilibration prior to collecting baseline values [arterial pH, Hct, mixed venous oxygen saturation (SvO₂), lactate, hemorrhage volume, Cardiac Output (CO), temperature, pulse rate (HR), Mean Arterial Pressure (MAP)]. Animals were bled over 30 min to reach an MAP of 30-41 mmHg. This pressure was maintained for an additional 30 min. Additional blood was removed if necessary to maintain the target MAP. Post-Hemorrhage (PH) values were recorded one hour after initiation of hemorrhage [3]. Of the five rabbits studied, one received crystalloid, three times the amount of blood removed (73.5 mL.kg⁻¹), one 25% BSA, 25% of blood withdrawn (6.1 mL.kg⁻¹), two 25% PEG-BSA, 25% of blood withdrawn (9.4 mL.kg⁻¹ and 5.5 mL.kg⁻¹) and one 75% PEG-BSA, 75% of blood withdrawn (21.2 mL.kg⁻¹). Post-Resuscitation (PR) values were taken 30 min, 1 hour and 2 hours after volume resuscitation. The results are displayed by plotting the raw data in each of the five animals for each variable.

The equipment used to measure arterial blood pressures, cardiac output, arterial blood gases and mixed venous oxygen saturation and the equipment used to measure the values recorded in Table 1 is as follows:

Cardiac Output (CO) and core body temperature was determined utilizing a Cardiomax II cardiac output computer system (Columbus Instruments International, Columbus, OH) with a thermistor catheter. Membrane transducers (Model 1290A, Hewlett Packard, Watham, MA) connected to a monitoring system (78353B Electro Cardio Graph monitor, Hewlett Packard, Andover, MA) were utilized to record systolic, diastolic and mean arterial blood pressure, Heart Rate (HR) and Central Venous Pressure (CVP). Arterial and venous pH (pHa, pHv) and partial pressures of O₂ (PaO₂, PvO₂) and CO₂ (PaCO₂, PvCO₂) were measured and bicarbonate (aHCO₃, vHCO₃) was calculated by a blood gas analyzer (Rapidlab Model 248, Bayer Corporation Diagnostics Division, Norwood, MA). Body temperature was maintained between 37-39°C by means of a heating pad and circulating warm air blanket (Bair Hugger Model 505, Augustine Medical Inc., Eden, MN) placed underneath and on top of the animal, respectively.

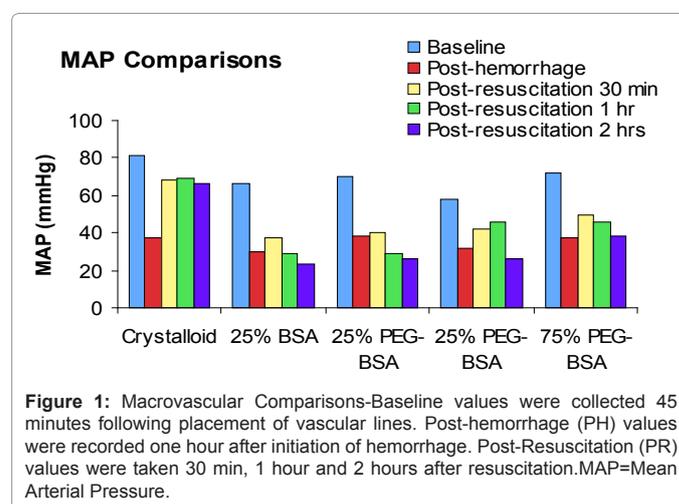
Results

Randomization was based on alternating the mode of resuscitation on successive days of study. The viscosity and Colloid Osmotic Pressure (COP) of the three-test resuscitation fluids used in this study are given in Table 1. See Figures 1-7 for the results in graphic format.

Body temperature remained stable in all five animals. The HR remained essentially at the baseline in all 25% hemorrhagic shock experiments but in 75% hemorrhagic shock studies where it returned to a value higher than the baseline value; propofol may have altered the natural HR response [4,5]. CO did not return to the baseline values when resuscitated with saline or 25% albumin, but returned close to the baseline with PEG-albumin both with the 25% hemorrhagic shock experiments and exceeded the baseline value in the 75% hemorrhagic-shock resuscitation model. In the saline resuscitated rabbit, MAP and SvO₂ returned to baseline values, the restitution of the baseline value

Sample	Colloidal Osmotic Pressure (mmHg)	Viscosity (cP)
0.9% Saline	<1.0	0.72
1% (SP-PEG5K)6-HSA	4.4	1.01
3% (SP-PEG5K)6-HSA	23.9	1.69
4% (SP-PEG5K)6-HSA	41.7	2.08
5% Albumin	21.4	1.15
25% Albumin	>200	5.0

Table 1: Plasma Expander like Properties of PEG-Albumin.



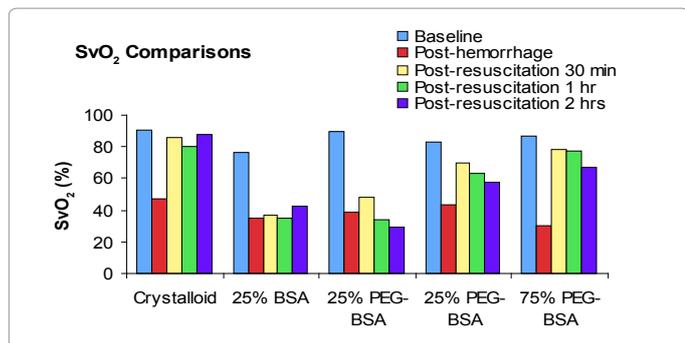


Figure 2: Macrovascular Comparisons- Baseline values were collected 45 minutes following placement of vascular lines. Post-hemorrhage (PH) values were recorded one hour after initiation of hemorrhage. Post-Resuscitation (PR) values were taken 30 min, 1 hour and 2 hours after resuscitation. SvO₂=oxygen saturation.

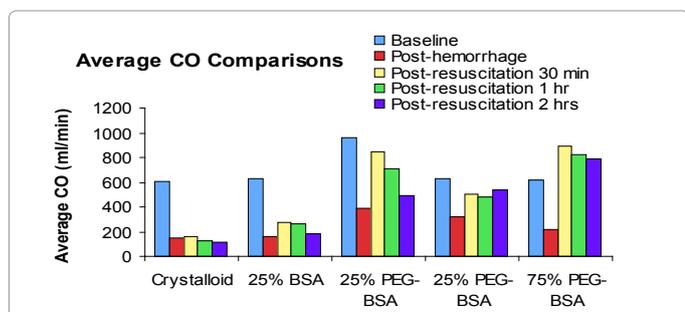


Figure 3: Macrovascular Comparisons-Baseline values were collected 45 minutes following placement of vascular lines. Post-hemorrhage (PH) values were recorded one hour after initiation of hemorrhage. Post-Resuscitation (PR) values were taken 30 min, 1 hour and 2 hours after resuscitation.CO=Cardiac Output.

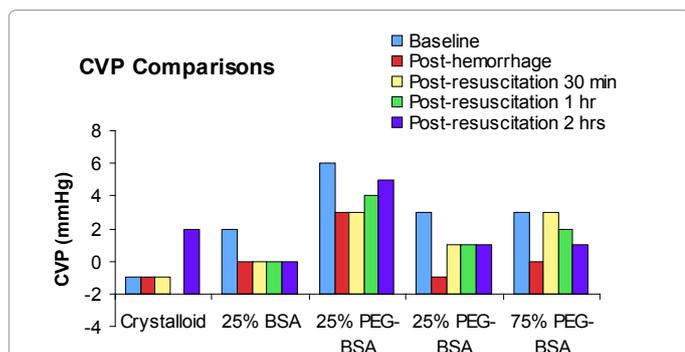


Figure 4: Macrovascular Comparisons-Baseline values were collected 45 minutes following placement of vascular lines. Post-hemorrhage (PH) values were recorded one hour after initiation of hemorrhage. Post-Resuscitation (PR) values were taken 30 min, 1 hour and 2 hours after resuscitation. CVP=Central Venous Pressure.

is least effective with albumin, while with PEG albumin the values returned to near baseline values.

The pH returned to baseline in all rabbits, where as lactate significantly increased and remained highly elevated relative to the baseline in all resuscitation studies. The different resuscitation fluids induce very distinct responses and reflect the need for more detailed studies to define the specific clinical applications where PEG-albumin may be a uniquely desirable material to use relative to others.

Discussion

The hemorrhage model used in the current study is the Wiggers model, gauging hemorrhage based on blood pressure as opposed to blood volume. Prior research has shown considerable support for the Wiggers model for studying hemorrhagic shock in both canine and rabbit models [6-8]. Fixed-pressure models, like the Wiggers model,

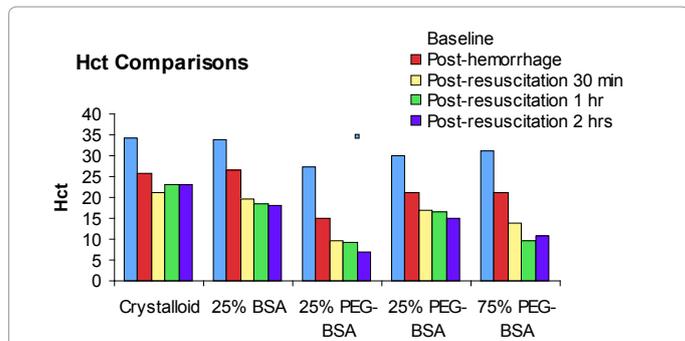


Figure 5: Macrovascular Comparisons- Baseline values were collected 45 minutes following placement of vascular lines. Post-hemorrhage (PH) values were recorded one hour after initiation of hemorrhage. Post-Resuscitation (PR) values were taken 30 min, 1 hour and 2 hours after resuscitation.Hct=hematocrit.

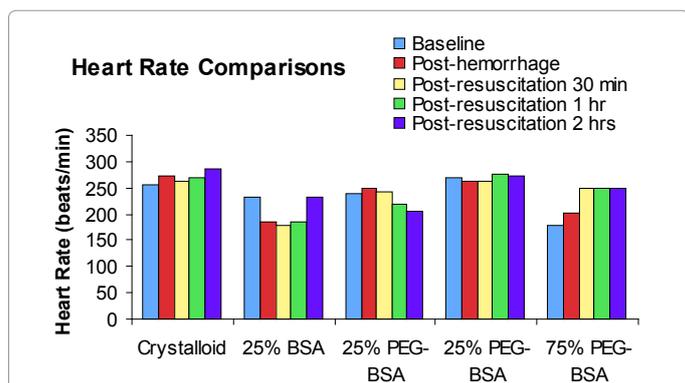


Figure 6: Macrovascular Comparisons-Baseline values were collected 45 minutes following placement of vascular lines. Post-hemorrhage (PH) values were recorded one hour after initiation of hemorrhage. Post-Resuscitation (PR) values were taken 30 min, 1 hour and 2 hours after resuscitation.Heart Rate Comparisons.

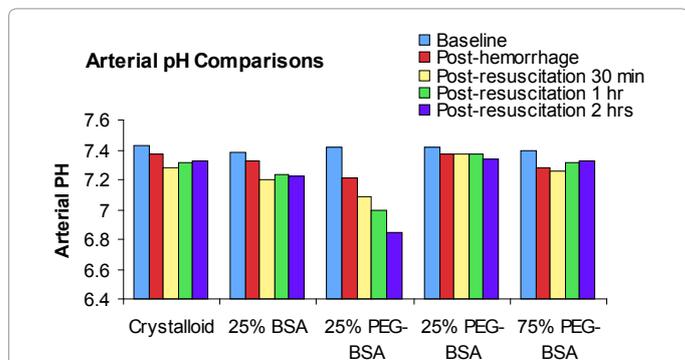


Figure 7: Macrovascular Comparisons-Baseline values were collected 45 minutes following placement of vascular lines. Post-hemorrhage (PH) values were recorded one hour after initiation of hemorrhage. Post-Resuscitation (PR) values were taken 30 min, 1 hour and 2 hours after resuscitation.Arterial pH Comparisons.

are favored over fixed-volume models because they have higher reproducibility and standardization [9]. Fixed-volume techniques may vary in response to a particular hemorrhage volume since different individuals have unequal total blood volumes [9].

Since the sample size is low, the results were interpreted descriptively by plotting the raw data in each of the five animals for each variable. As a general trend, it appears that saline resuscitation improved MAP and SvO₂, but HR, Hct, pH and lactate improved with pegylated albumin solutions, suggesting improved macro vascular resuscitation with saline and possibly improved microvascular resuscitation with the colloid solutions, although this was not specifically tested (Figures 1,2,5-7,9).

Although the results of our study do mirror the findings of similar studies [1], it is important to keep in mind that our results are based upon pilot data. Because the sample size is small, statistical analysis was not an option, so statistical significance could not be assigned to the results. Improvements on experimental design should include larger sample sizes for each resuscitation agent being tested.

As a general trend, the non-PEG crystalloids appear to improve macrovascular resuscitation, while the PEGylated solutions improve microvascular resuscitation. These findings are similar to the work done by Cabrales et al. on extreme hemodilution: “PEG-conjugated

albumin and Hb [hemoglobin] lead to physiological microvascular and systemic conditions that are closer to baseline than using non-PEG materials such as albumin and Dx 70” [1].

Utilization of protein-based drugs for hemorrhagic resuscitation presents a challenge because they are vulnerable to immunogenic reaction and proteolytic degradation [2]. Recent advances have allowed for the synthesis of solutions of PEGylated therapeutic proteins solutions that are relatively stable at neutral pH and are therefore kept in circulation longer than the corresponding non-PEGylated proteins. This has resulted in the development of PEGylation as a novel technology to modulate the pharmacokinetics of protein, peptide and small molecular weight drugs [10].

PEGylated bovine Hb was developed by Enzon as a potential blood substitutes in view of the multiple beneficial influence of PEGylation on proteins. Another intermolecularly crosslinked Pyridoxylated Hb PEGylated (PHP) was developed by Ajinomoto. The PEGylated derivatives of Hb, particularly Enzon PEGylated Hb was distinct from other derivatives of Hb in that it was vasoinactive and nonhypertensive. The unique distinguishing properties of PEG-Hb as compared to that of other conventional derivatives of Hb previously developed as blood substitute are very high hydrodynamic volume (molecular dimensions) disproportionate to the molecular mass, high viscosity and higher colloidal osmotic pressure. These are the properties of colloidal plasma expanders, and the PEG-Hb has been accordingly referred to oxygen carrying plasma expanders. Accordingly inducing the plasma expander like properties to Hb by PEGylation has been advanced as a new design strategy to attenuate the in vivo hypertensive activity of acellular Hb and to develop oxygen therapeutics.

Designing the optimized PEG-Hb in terms of the pattern of PEGylation and chemistry of conjugation has been a subject considerable interest in recent years. Conjugation of PE-chains to Hb, a protein or four subunits, two copies each of the two-polypeptide chains (α and β chains), leads to a weakening of the interdimeric interactions of the protein. However, if Hb is PEGylated using extension arm facilitated PEGylation (EAF-PEGylation) protocol wherein spacer arms are introduced between the side chain functions of Hb and PEG-chains, the influence of PEG-chains on the interdimeric interactions of Hb is attenuated and the stability of Hb and EAF-hexaPEGylated Hb is essentially the same.

Delineating the influence of the pattern of PEGylation and of the chemistry of conjugation on the properties of PEG-Hb as a resuscitation fluid has been the subject of primary interest in developing the blood substitutes. Though the original PEG-bovine Hb carried nearly 10 copies of PEG-5K chains coupled to the amino groups through urethane linkage, the EAF-hexaPEGylated (using PEG 5K) Hb was found to be vasoinactive. Indeed this approach of PEGylation of Hb has been taken up for the commercialization of PEG Hb as oxygen carrying plasma expanders. The albumin analogue of PEG-Hb has all the plasma expander like properties of PEG-Hb but not the heme induced toxicity and is referred to as non-oxygen carrying plasma expanders. The PEGylated albumin is a new class of plasma expander, superior to hetastarch and albumin as reflected in the studies in hamsters. PEG-albumin lowers the transfusion trigger and also acts as a vasodilator [10].

These solutions of PEG albumin and PEG Hb are an important class of resuscitation fluids useful in arresting hemorrhage. The choice between the two will probably dictated by the level of blood loss and hence the need for the restituting the supply of oxygen besides replacing

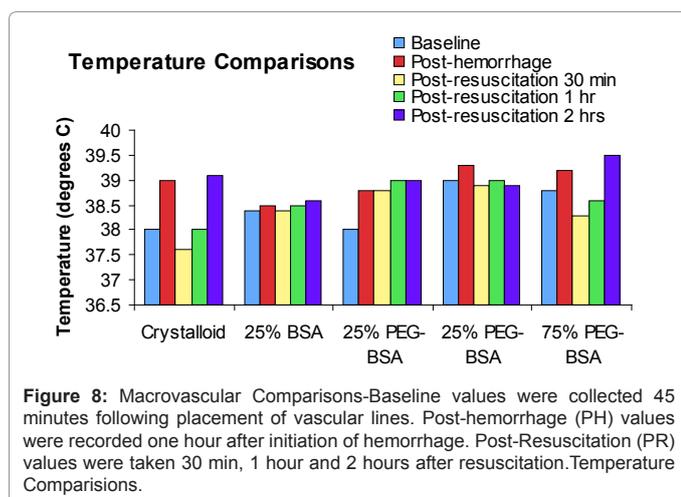


Figure 8: Macrovascular Comparisons-Baseline values were collected 45 minutes following placement of vascular lines. Post-hemorrhage (PH) values were recorded one hour after initiation of hemorrhage. Post-Resuscitation (PR) values were taken 30 min, 1 hour and 2 hours after resuscitation. Temperature Comparisons.

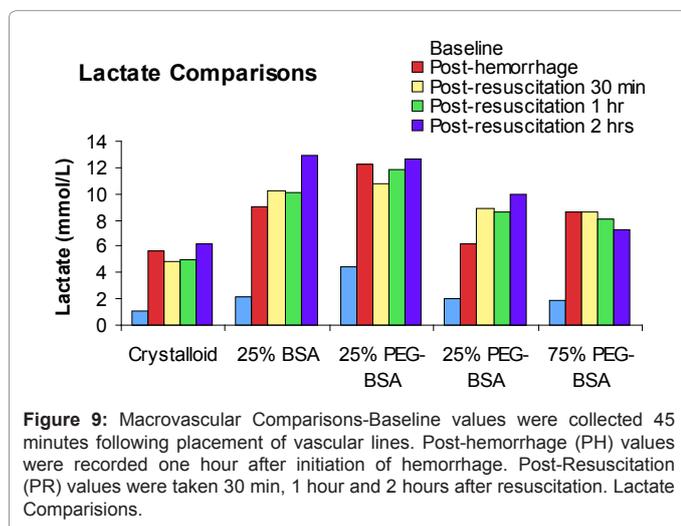


Figure 9: Macrovascular Comparisons-Baseline values were collected 45 minutes following placement of vascular lines. Post-hemorrhage (PH) values were recorded one hour after initiation of hemorrhage. Post-Resuscitation (PR) values were taken 30 min, 1 hour and 2 hours after resuscitation. Lactate Comparisons.

lost volume. The PEG represents the most popular synthetic polymer that has been studied, it is nontoxic, nonimmunogenic, highly soluble in water, and FDA approved [2]. These characteristics make PEG-albumin and PEG-Hbs solutions deal for the treatment of hemorrhagic shock. The absence of toxicity with MP4, a hexaPEGylated Hb carrying six copies of PEG-5K chains conjugated using extension arm chemistry in recent clinical trials has suggested the potential of PEG-albumin as plasma expander, in situations where in blood loss needs only the replacement of the lost blood volume, but not the restitution of the oxygen carrying capacity.

The stock solution of the primary resuscitation fluid, hexaPEGylated albumin is at 4 gm% in PBS. This solution has a viscosity (2.2 cP) that is significantly lower than blood and COP that is nearly twice that of plasma. Accordingly, the replacement of lost blood by this resuscitation fluid will make the circulating fluid less viscous relative to the original blood, irrespective of the volume of the blood replaced by this resuscitation fluid. Since the COP of the PEG-albumin solution is higher than that of plasma, the COP of the circulating fluid after the replacement of the blood volume will be a function of the volume of the PEG-albumin solution used to replace the lost blood. The final COP of the circulating fluid is not a direct correlation of the dilution factor as the COP of the solution is an exponential correlate of the concentration of PEG-albumin in the solution. On the other hand the 25% albumin solution is a high viscosity (5, 0 cP) resuscitation fluid and also has a high COP (>200 mm Hg) solution as compared to PEG albumin solution. 25% hemorrhagic shock resuscitation with this resuscitation fluid (25% HSA) is a system at the high viscosity end. On the other end of the viscosity spectrum is saline, a very low viscosity (0.72 cP) and low COP resuscitation fluid. Thus in comparing the responses of the three test resuscitation fluids, the influence of the differences in the plasma expander like properties of these solutions, in particular the viscosity, on the vascular responses is addressed in these studies.

It may be noted that the viscosity of a 4 gm% solution PEG-albumin is lower than that of blood as well as that of 25% BSA. Thus in replacing the shed (lost) volume of blood in the studies does not result in the restitution of the original viscosity of blood. Either 25% or 75% of the blood has been replaced. The significant decrease in the hematocrit with time in the 75% PEG Alb animal represents uniqueness of this resuscitation fluid to maintain the macrovascular function very effectively even with low levels of RBC and even when metabolic functions are severely compromised. The high efficacy of PEG-albumin as plasma expander may also be a consequence of the higher colloidal oncotic pressure of this test solution (as compared blood) which can facilitate the auto transfusion of liquid from tissues, and the resulting improvement in the functional capillary density. PEG-albumin has also been shown to be a vasodilator. This activity is apparently a result of interaction of PEG-albumin with RBC to enhance the shear thinning and induced an increase in endothelium NO production i.e., PEG albumin is a low viscosity NO producing plasma expander.

In conclusion, HexaPEGylated albumin [(SP-PEG5K)₆ Albumin] generated using Extension Arm Facilitated PEGylation is a new class of hybrid semisynthetic biopolymer with excellent plasma expander-like properties and accordingly has been advanced as a novel

resuscitation fluid. The responses to the resuscitation by the three fluids in rabbit hemorrhagic shock models is distinct and expose the general trend for the application of PEG-BSA at low concentrations as a novel resuscitation fluid. It appears that resuscitation with 0.9% sodium chloride, improves only MAP and SvO₂ suggesting improved responses. The PEG-BSA as well as albumin, improved MAP, SvO₂, HR, pH, and lactate, the PEG-BSA accomplishing this with very low levels of materials relative to albumin. These findings, while limited by low number of animals studied, suggest that this novel resuscitation fluid requires further study in larger groups and larger animals to validate these preliminary results.

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