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14. ABSTRACT

The effectiveness of polyethylene glycol (PEG) based low volume resuscitation (LVR) solutions is dependent on polymer size, which correlated with their distribution in the microcirculation. Shocked rats resuscitated with PEG-20k all survived 24 hours (100%) compared to saline volume controls (0%) and had brain function scores comparable to sham controls after recovery from shock. PEG-20k was mainly excreted by the kidneys with a half-life of about 6 hrs. Maximum PEG-20k blood levels (3 mg/ml) were 3 times lower than the lowest dose that produced a mild coagulopathy in ex-vivo blood testing using TEG in volunteers or trauma patients (10 mg/ml). Further coagulation and platelet function studies suggest the mild coagulopathy with higher doses of PEG LVR solutions is due to nonspecific platelet passivation to produce a slight thrombasthenia with very mild effects on fXIII induced fibrin cross bridging (at 10 mg/ml). In acute swine with lethal hemorrhage, LVR using PEG-20k significantly increased survival to the 4 hrs post-reperfusion end point with normal blood pressure and lactate. Resuscitation with bequal voilumes of LR, Hextend, or autologous whole blood showed much worse outcomes. All pigs died within 90 minutes with very high lactates and lethal hypotension. Twenty-four hour survival was 100% in PEG20k treated swine and 0% in Hextend or whole blood. Survivors had normal lactate and neurological function scores.

15. SUBJECT TERMS
Polyethylene Glycol, PEG-20k, Resuscitation, low volume resuscitation, shock survival

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1. **INTRODUCTION:** Earlier work from our lab has described a new mechanism of tissue reperfusion injury in shock that is similar to what we had described many years earlier for the preservation of organs for transplantation. Specifically, the metabolic cell swelling of cells that occurs when ATP levels are exhausted during organ ischemia during shock are much more important than previously thought. As energy dependent cell volume control mechanisms are lost during shock, sodium, chloride, and water move into the cell and tissues, which causes metabolic cell swelling. This secondarily compress capillary networks perfusing the tissues and causes further ischemia. More importantly, the compressed microcirculation leads to poor and incomplete resuscitation, which aggravates the problem further. To fix this (and test the hypothesis), we administered cell impermeant molecules to shocked animals to reduce cell swelling by osmotically holding water outside of the cell (because these molecules are impermeant to the cell membrane). This decompresses the capillaries and allows tissue perfusion under low flow conditions and improves outcomes. One complex cell impermeant used was a polymer of polyethylene glycol (PEG-20k), which produced logarithmically better results than any other solution, including standard clinical solutions and our standard impermeant solutions. Since the PEG-20k active molecule is a polymer and since other polymers like hydroxyethyl starch cause coagulopathies, we determined the effects of PEG-20k based LVR solutions in an ex-vivo model of clotting blood using thromboelastography (TEG) analysis of human blood obtained from healthy volunteers and from trauma patients in our Emergency Department. We found that 10% dilutions of PEG-20k LVR solutions does cause a slightly hypocoagulable state that is dose dependent. How this solution affects coagulation and clot formation in-vivo in severe hemorrhagic shock is unknown. Therefore, our objectives for this project were to **1.)** Determine how this new LVR solution performs in a rodent SURVIVAL model of lethal hemorrhagic shock and low volume resuscitation, **2.)** Determine the mechanisms of action of PEG-20k LVR solutions on the dose dependent changes in coagulation and platelet function on ex-vivo clotting whole human blood, **3.)** Determine the effects of PEG-20k solutions on coagulation and platelet function in a pre-clinical porcine model of severe hemorrhagic shock (acute model) and to determine basic pharmacokinetic behavior of PEG-20k in this setting, and finally, **4.)** To determine the effects of PEG-20k LVR solutions in porcine survival models and in rodent uncontrolled hemorrhage models.

2. **Keywords**: Crystalloid IV fluids, hemorrhagic shock, osmotic effects, oxygen debt repayment, tissue swelling, Coagulation, Platelet Function, TEG, neurological deficit, survival

3. Accomplishments

What were the major goals of the project? The major goals of the project were:

I. <u>To identify mechanisms of action of PEG-20k in low volume resuscitation (years 1 and 2)</u>

a.) Determining the effects of PEG polymer size on LVR outcomes to support the hypothesis that only specific sizes of PEG polymers work because they define the unique osmotic reflection coefficient needed to produce the double osmotic gradient in the microcirculation.

b.) Reconstitute the PEG-20k resuscitation effect by using both an oncotic agent with a cell impermeant to validate the double osmotic gradient hypothesis

c.) Determine the effects of PEG-20k in an uncontrolled hemorrhagic shock model to see how the solution performs in uncontrolled bleeding settings

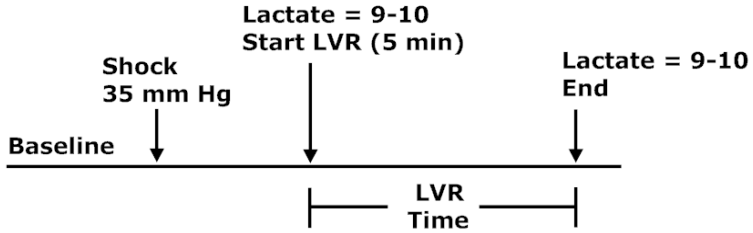
II. To translate effects of PEG-20k to a pig model of controlled and uncontrolled hemorrhagic shock (Years 2 and 3).

III. To assess PEG-20k based LVR solutions on coagulation and platelet function in blood from volunteers and trauma patients (year 3)

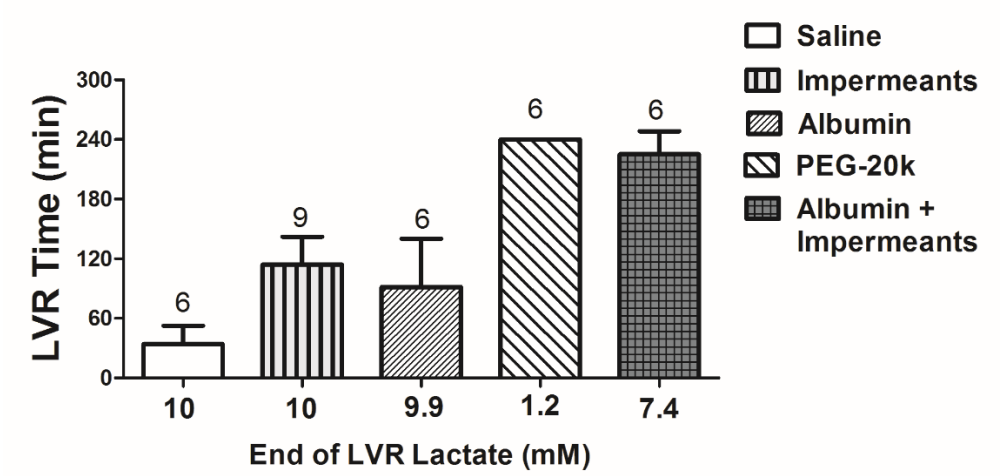
What was accomplished under these goals?

Effects of adding impermeants with colloids on LVR times in shocked rats, relative to PEG-20k. Five groups of rats were shocked, given an LVR solution (volume equal to 10% of the calculated blood volume), and the LVR time determined. LVR time is determined as the time from the start of LVR, which is triggered by lactate between 9-10 mM, until the time when lactate again reached the 9-10 mM limits after LVR. This is described visually in Fig 1.

Figure 1



The groups tested included animals resuscitated with LVR solutions containing saline (negative control group), PEG-20k (positive control), and test solutions consisting of an impermeant alone (gluconate), a colloid alone (albumin), and a combination of an impermeant and colloid (gluconate with albumin). The LVR times are shown in Fig 2

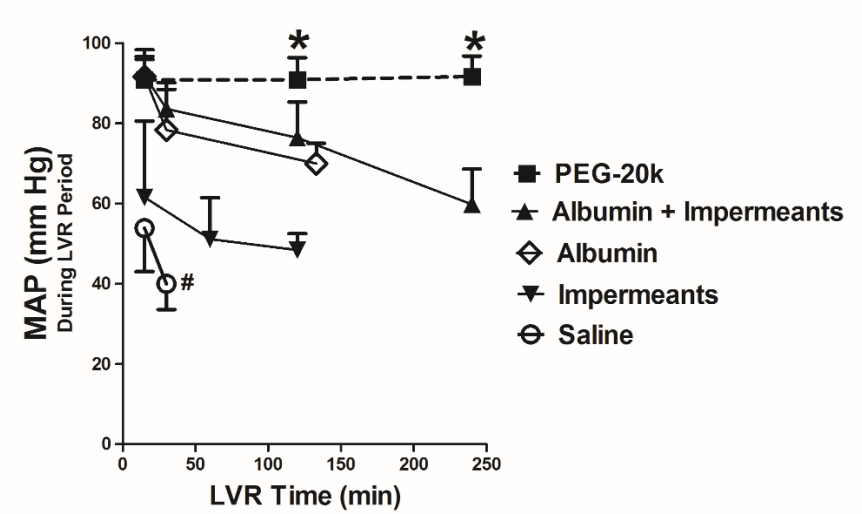


Low volume resuscitation with an impermeant increased LVR time (tolerance to the low volume state) 4 fold from 30 to 120 minutes. This was about the same response seen with the colloid albumin. These responses fall far short of the 8 fold increase in LVR time observed with PEG-20k. However, combining both albumin and gluconate increased LVR times equivalent to what was observed with PEG-20k, suggesting that PEG-20k may work, in part, by establishing two osmotic gradients in the microcirculation similar to the two gradients established by the combined solution group in this study. These results support the hypothesis but do not exclude other mechanisms of action for PEG-20k. Furthermore, the true LVR responses between the combined group (LVR solution with both gluconate and albumin) and the PEG-20k group may have been significantly different had we not stopped the LVR period at 240 minutes because the end lactates were significantly different between those groups. Specifically, the lactate in the combined LVR solution group was close to the 9 mM limit while the

lactate in the PEG-20k LVR group was far from the limit and continuing to fall towards baseline. This suggests that the true LVR times in the PEG-20k group would be much higher than the true LVR time in the gluconate/albumin group.

Effects of adding impermeants with colloids on arterial blood pressure in shocked rats, relative to PEG-20k. Similar to the LVR response, we observed improvements in mean arterial blood pressures when both impermeant and colloid solutions were combined compared to using them separately. The response was not as good as seen with PEG-20k however. The results are shown in Fig 3.

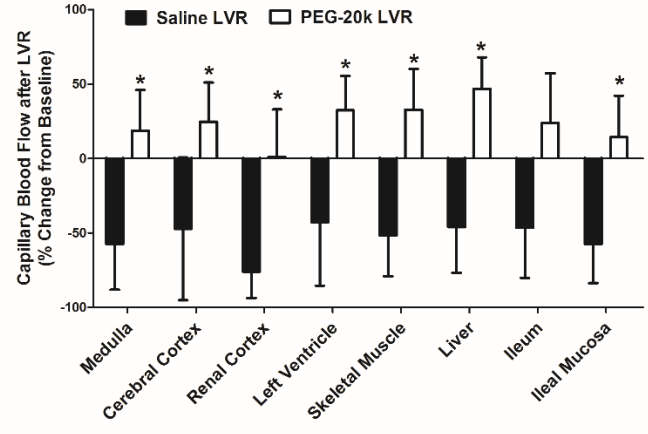
Figure 2



These results support the hypothesis that impermeants draw water out of cells and into vascular spaces, which would also lead to an increase in MAP during the LVR period. This drives blood flow in the tissues for oxygen transfer in the low volume state and increases LVR times.

Effects of PEG-20k LVR on capillary blood flow in shocked rats, relative to a saline volume control. To further support the hypothesis that PEG-20k increases LVR times by moving isotonic water from the extravascular compartment into the vascular space, we measured direct capillary blood flow changes in the LVR period with PEG-20k resuscitation. Capillary blood flow was measured in the LVR period after either saline or PEG-20k resuscitation using colored microspheres. Regional capillary flows are shown in Fig 4 for both groups in many important vascular beds.

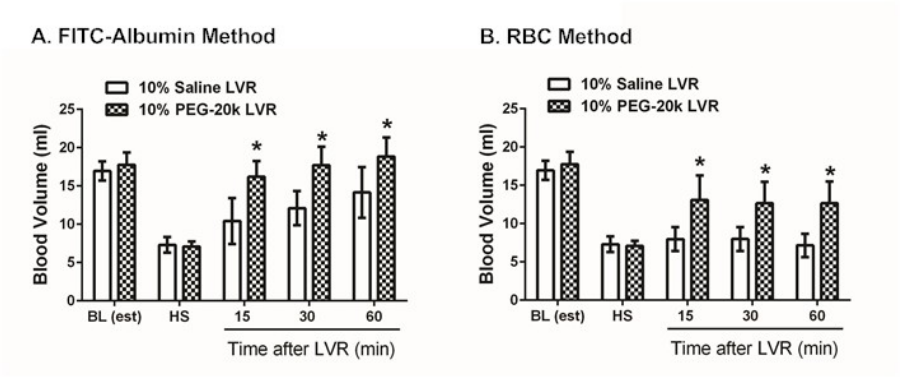
Figure 3



Capillary blood flow was significantly higher during LVR in all vascular beds when PEG-20k is added to the LVR solution, relative to the saline vehicle volume control. Again, this further supports our mechanistic hypothesis of an osmotically driven refilling of tissue capillaries that transfers oxygen in the tissues during the low

volume state and drive down lactates to increase observed LVR times. In essence, PEG-20k LVR prevents the shock-induced drop in capillary blood flow in important vascular beds. Presumably by increasing the intravascular volume. To address this further, we measured directly the volume in the intravascular space during shock and LVR in these two groups by using two different indicator dilution techniques involving FITC-labeled albumin dilution and red blood cell (hemoglobin) dilution. Figure 5 shows these results.

Figure 4



PEG-20k containing LVR solutions significantly expanded the intravascular volume during shock when the saline vehicle did not. This directly supports the hypothesis on the mechanisms of how these unique polymers work in our shock model.

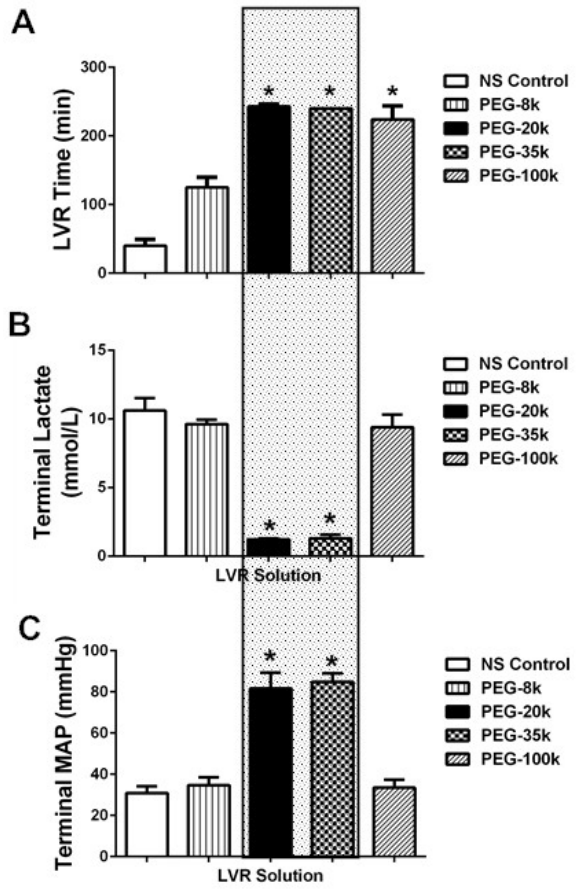
PEG Polymer Size and effects on low volume resuscitation (LVR) from shock.
We hypothesized that PEG-20k is effective because eit has a unique molecular size and radius that allows it to partially but unequally partition in both the capillary and interstitial space while remaining impermeant to the intracellular space. Conversely, it this is true, the larger and smaller size polymers should be less effective. We used (4) sizes of PEG polymers in our standard rodent shock and low volume resuscitation model in acute experiments using low volume resuscitation (LVR) times as the primary outcome. The PEG polymer sizes and groups of rats included;

- Normal Saline (NS) volume control (10% of estimated blood volume) used for LVR
- PEG-8k (10% estimated blood volume) used for LVR
- PEG-20k (10% estimated blood volume) used for LVR
- PEG-40k (10% estimated blood volume) used for LVR
- PEG-100k (10% estimated blood volume) used for LVR

All solutions were used at a 10% concentration of the polymer in saline and administered in a volume of 10% of the estimated blood volume when given for resuscitation. This is equivalent to 500-ml volume for resuscitation of an adult male patient. The protocol for the shock and resuscitation of the rats is shown diagrammatically in Figure 1.

After general anesthesia induction, rats were surgically implanted with vascular catheters and bled through the arterial line until blood pressure reached 35 mm Hg. Pressure was held at this level to increase the oxygen debt, as indexed by the rising plasma lactate level. Once the plasma lactate target of 9-10 mM was reached, a low volume resuscitation crystalloid was given I.V. at 10% of the estimated blood volume over 10 minutes. The blood volume removed to achieve this debt level was typically about 55-60% of the total blood volume, which is a lethal hemorrhage for the volume control (NS) and many other crystalloids such as 10% albumin solution, Hypertonic saline solution, or 6% Hextend solution. The LVR times of each of the groups in this experiment are shown in Figure 6. The higher the LVR time, the more tolerant the rat to the same level of shock.

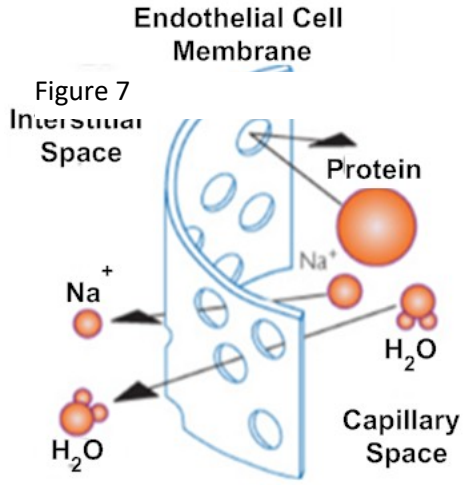
Fig 6



The LVR time (panel A) is an index of the tolerance of the rats to the low volume state. The more metabolically tolerant they are, the longer it takes them to re-build oxygen debt after a low volume crystalloid resuscitation. The time when they rebuild debt to a lactate of 9 mM again is the time that they require definitive resuscitation to prevent death. Thus, all PEG containing LVR crystalloid solutions significantly prolonged this tolerance period, relative to the volume control with normal saline. A "sweet spot" in PEG polymer molecular weight size was reached between 20-40 kDa where maximum LVR times were seen and where polymers above or below that range were not as protective. This is further exemplified in the data in Figure 6 by comparing the terminal lactates (panel B) and the end systolic blood pressures in the polymer size groups (panel C). Again, PEG-20 to 40k had the lowest end lactate levels and the highest end systolic blood pressures compared to lower polymer sizes (PEG-8k) and higher weights (PEG-100k). It should also be noted that the LVR times for the 20 and 40k PEG polymers were arbitrarily cut off at 240 minutes so their real LVR times were likely much higher, especially since their lactates after 240 minutes were at baseline values (1.2 mM), which are far below the values needed to trigger the end of the LVR time (9-10 mM). Therefore, while all PEG polymer sizes tested were much better than saline, the dramatic effects seen previously are in fact dependent on a strict molecular weight range, which we hypothesize establishes the mechanism of action of this new impermeant class polymer in shock and low volume resuscitation. More specifically, we hypothesize that this strict molecular weight range allows the PEG polymers to have, at equilibrium, partial and unequal excursions of the molecules outside of the capillary space while maintaining many molecules in the capillary space too. This would be defined by the osmotic reflection coefficient that was next characterized in healthy anesthetized rats.

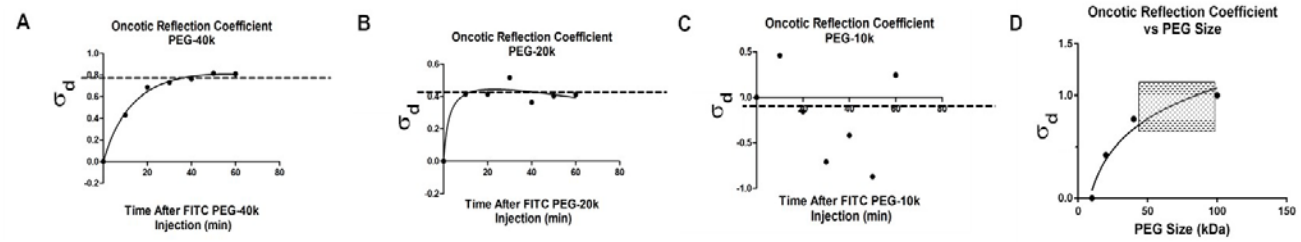
To precisely test this hypothesis and measure the distribution of polymer molecules outside the capillary in the interstitial space versus inside the capillary space, we measured the osmotic reflection coefficients for each of these polymer sizes. A reflection coefficient of 0 means the molecules are not reflected back into the capillary at all and are free to equilibrate evenly between the capillary space and the interstitial space (Figure 7). On the other hand, a reflection coefficient of 1of 1 means all of the molecules are reflected back into thethe the capillary and are blocked from diffusing out or blocked from being "dragged" out of the capillary pores by solvent (water) convection. The osmotic reflection coefficient is calculated by: σ_d= 1- [L/P], where where [L/P] is the lymph to plasma concentration ratio of the test compound at high lymph flow rates (volume loading) conditions. The test polymers used were tracer polymers containing FITC so activity in lymph and plasma is monitored by detecting fluorescence using an excitation-emission spectrofluorometer. The ramifications of having a unique solute that is both impermeant to cells AND

Figure 7



unequally partitioned into both the interstitial and capillary spaces, i.e., has a reflection coefficient greater than 0 but less than 1 is that two distinct osmotic gradients are established outside of the cell to drive the transfer of free water from the metabolically swollen cell into the capillary space where it belongs. This water transfer then decompresses capillary beds by decreasing the transmural pressure on them that tends to reduce their effective radius and limit flow while increasing capillary volume and pressure that supply the driving force for capillary flow. Figure 8 shows the results of the osmotic reflection coefficient studies with polymer sizes of PEG-8k, PEG-20k, PEG-40k, and one experiment with PEG-100k.

Figure 8



In Figure 8, the osmotic reflection coefficients are shown over time after the injection of the fluorescently labelled tracer PEG polymer. It generally takes 10-20 minutes to achieve a steady state as seen by the flat lines, which represent the true osmotic reflection coefficients. No raw data are shown for PEG-100k because the commercial material was found to be contaminated with smaller labeled polymers besides 100k so we had to purify the material by size exclusion filtration before use. This is why the curve in Panel D shows a shaded box from PEG 40k to PEG-100k because we have very limited data and the results between those points is less certain. In panel A, the partitioning of PEG-40k is shown to be about 0.8, which means that for every 4 molecules that stay in the capillary space, 1 moves into the interstitium (and 0 go into the cell). Panel B shows the same coefficient data for PEG-20k and recapitulates the osmotic reflection coefficient of 0.4, which means that for every 5 molecules of PEG-20k that stay in the capillary space, 2 wander out into the interstitial space (and 0 enter the cell). Figure 4C show the partitioning for PEG-8k with a coefficient of essentially 0 (negative numbers are actually unachievable and represent sampling error). This means that for any number of PEG-8k molecules that stay in the capillary, the same number migrate into the interstitial space (and 0 enter the cell) because the material is freely permeable to the capillary membrane. Finally, for PEG-100k, our currently limited data suggest a coefficient around 0.9-1.0, which means that almost all of the PEG-100k molecules are confined to the capillary space with only limited numbers (<10%) being partitioned into the interstitial space (and 0 moving into the cell). This material behaves as a classic colloid. These coefficient data, together with the shock outcomes data (Figure 6), indicate that the most effective PEG polymers are between 20-40 kDa and possess a reflection coefficient between 0.4-0.7. This by definition describes the establishment of multiple osmotic gradients by the most effective PEG polymer sizes and supports our hypothesis regarding conditions for effective energy independent unidirectional osmotic water transfer from cell to capillary.

Because the polymer sizes used in these studies are large, the actual numbers of molecules and osmotically active particles partitioning into these spaces is relatively small. So, the osmotic gradients established are relatively small (mOsM concentrations in the single digits). However, the relative attraction for water molecules by PEG polymers is large as they are known to form multiple water “shells” around the PEG polymer backbone (Figure 9). This likely explains why relatively small osmotic gradients of PEG have such huge water transfer properties.

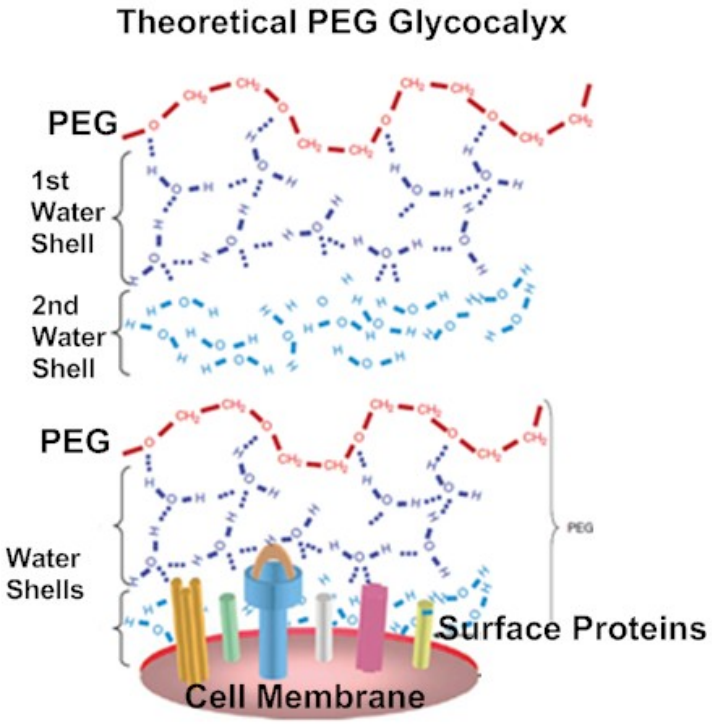
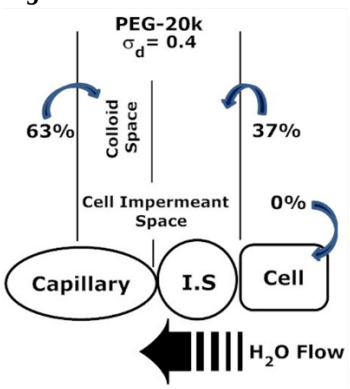
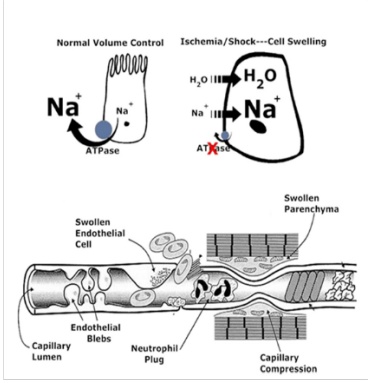


Figure 9
As is also shown in Figure 9, the PEG polymers are capable of binding to and possibly repairing the endothelial cell glycocalyx. Since the glycocalyx is likely injured in severe shock and low volume resuscitation, we will in the future explore whether some biological effects of PEG-20k based LVR solutions are partly attributable to rebuilding and rehydrating the damaged glycocalyx.

At this point, we have identified a likely hypothesis to explain how PEG-20k works in LVR. Based on our presented data, we believe that the unique osmotic reflection coefficient for PEG-20k establishes 3 osmotic gradients in the microcirculation that efficiently and non-energetically moves metabolic water out of the cells and into the capillary spaces. The result is to prevent cell swelling injury, decompress the microcirculation, reload the capillaries, and dramatically improve capillary flow and oxygen transfer under very low volume conditions. **Figure 10** shows this diagrammatically.

Figure 10





Effects of PEG-20k LVR solutions on shock survival and neuro-cerebral function
We have clearly demonstrated in acute experiments in the rodent and pig model how PEG-20k LVR solutions increase the tolerance to the low volume state 20 fold over saline volume controls, based on the low volume resuscitation time. In the next study we determined if there was a translational survival benefit to being resuscitated with PEG-20k LVR as indexed by next day survival in rats treated with PEG-20k compared with the saline controls. The table shows the survival increase from 0% (saline) to 100% (PEG-20k), neuro-deficit scores that remain normal for PEG-20k treated rats (0 = normal, 500 = brain dead), urine production, day 2 mean arterial pressures (MAP), and day 2 lactates. The rats experiencing severe lethal hemorrhagic shock are not different compared to sham operated rats using these outcomes. But they are vastly improved over the shocked rats

receiving saline (0% survival), In addition to surviving the lethal shock, PEG-20k resuscitated rats had normal brain function (NDS).

TABLE 1: Rodent 24 hour survival data after lethal hemorrhagic shock in PEG-20k and normal saline (NS) resuscitated groups. UOP=Urine output, MAP=mean arterial pressure.

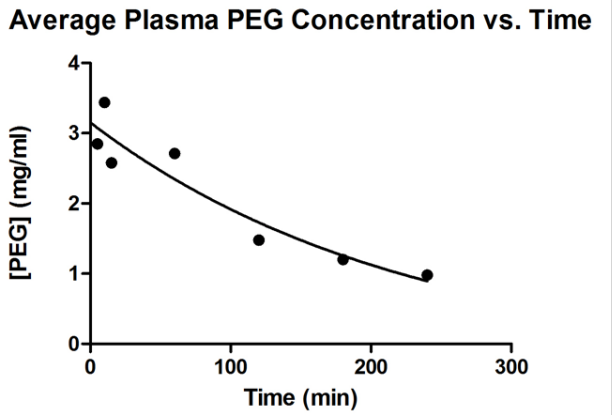
Group (n)	Survival (%)	NDS	UOP (cc/kg/hr)	Day 2 MAP (mmHg)	Lactate (mmol/L)
PEG 20k (8)	100	64	2.8	86	2.7
Sham (1)	100	40	5.11	109	2.8
NS (5)	0	N/A	N/A	N/A	N/A

NDS - Neurologic Deficit Score: 0 – normal, 500 – brain dead. Values are given as means.

Plasma elimination of PEG-20k after resuscitation from lethal hemorrhagic shock

The elimination and blood levels of PEG-20k were monitored in these animals over 24 hours to determine the half-life of elimination and to determine the exact blood levels immediately after resuscitation. These initial blood levels represent peak levels and are the maximum levels use to compare our ex-vivo platelet function and coagulation study results that were conducted in parallel studies. PEG-20k is administered as a theoretical 10% dilution of the calculated circulating blood volume. Therefore, administration of a solution with a concentration of 100 mg/ml of PEG-20k should dilute to 10% immediately after administration and achieve theoretical levels of 10 mg/ml. Therefore, we tested PEG-20k in whole blood at 10 mg/ml for coagulation and platelet function using Thromboelastography (TEG) and other platelet and coagulation factor specific assays (described in the next section and in two appended manuscripts). The results of the blood level study are shown in figure 11 and are derived using a FITC-labelled PEG-20k probe to detect blood levels over time in shocked rats.

Figure 11



Pharmacokinetics (PK)

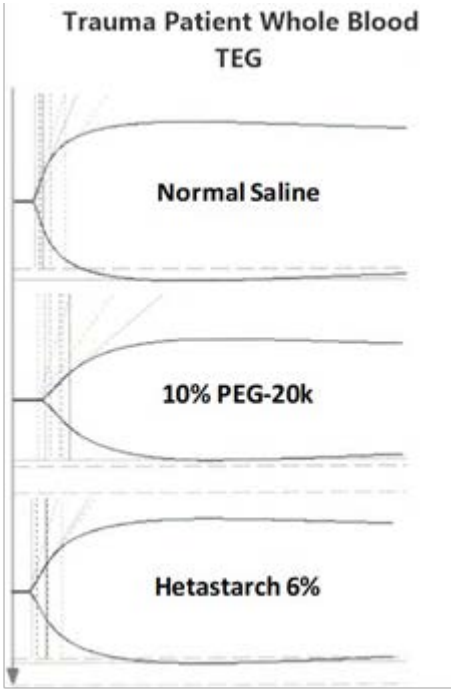
$t_{1/2}$ = 156 min

Eliminated in the urine

Important features to note are the initial blood levels (3 mg/ml) and the half-life of over 2 hours. The initial blood levels are **3 times lower** than the blood concentrations used in the coagulation studies. This will be important to remember when those results are presented. Also, the half-life is rather short for a single administration agent so concerns about prolonged exposure and toxicity are alieved.

Mechanisms of action of PEG-20k and related polymers on coagulation and platelet function in human blood: Acute resuscitation from severe hypovolemic shock with small volumes of PEG-20k containing crystalloids is highly effective. However, since the active agent is a polymer and since starch based solutions (Hextend) are also polymers that cause coagulopathies, there has been concern that these solutions may also cause coagulopathies. In ex-vivo testing of whole blood from healthy volunteers or from trauma patients immediately on arrival to our ED (before they have been transfused), 10% PEG-20k LVR solutions caused a hypocoagulative state as seen on TEG analysis (Figure 12). This effect is similar to the hypocoagulative state caused by 6% Hextend. The TEG analyzes coagulation from the chemical component and the cell (platelet) component in aggregate because it measures the physical dynamics of the formation of a blood clot. The reduced width of the chart, known as the MA, is 80% due to platelet function effects while the changes in the slope of the shoulders, as measured by the k and slope function on TEG, indicate possible changes in clotting factors, fibrinogen, or dilution of the chemical components. The net effects may be due to interference by PEG-20k on any one or all of these components. The aim of this quarters work was to dissect out these mechanisms.

Figure 12



Since the angle and k values were altered with PEG, explored if fibrinogen and von Willebrand factor concentrations were altered. As seen in figure 13, PEG-20k solutions did not change these levels, relative to the saline dilutional control, which suggests that the altered amplification and propagation phase of the clotting process were not altered by lowered fibrinogen or vWF

Figure 13

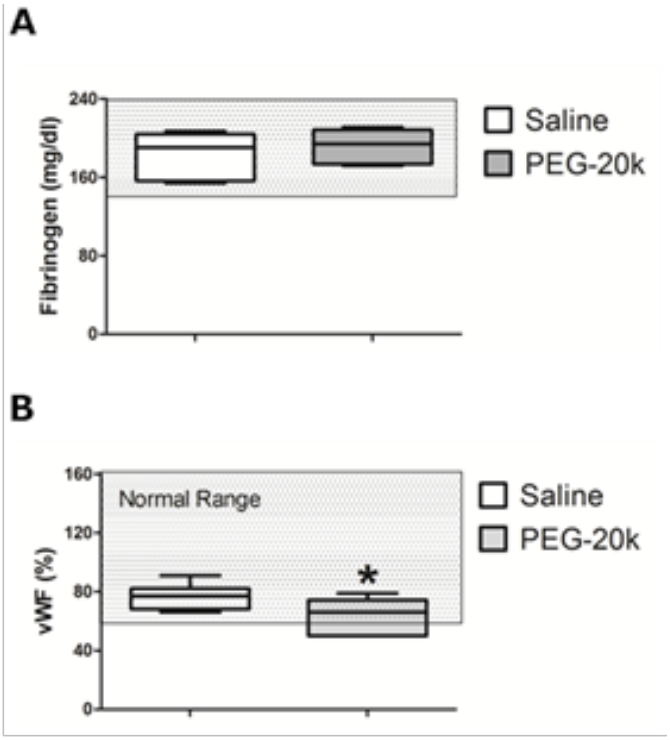
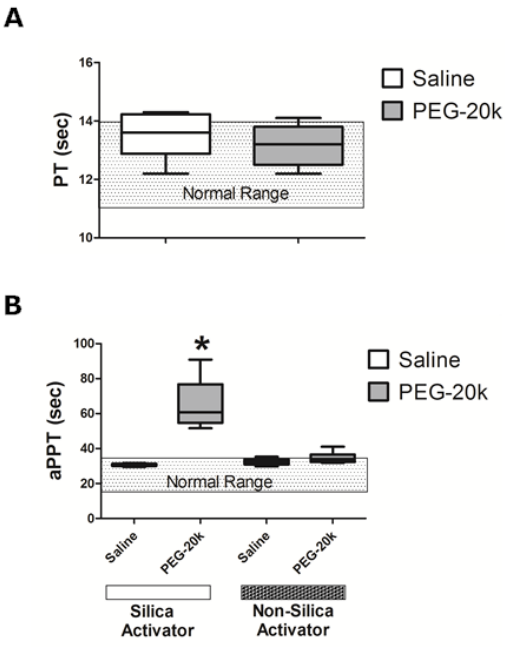
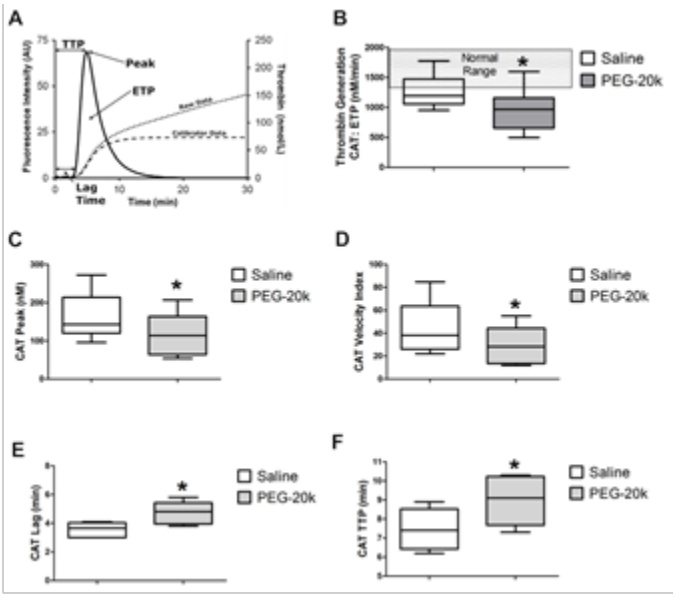


Figure 14



To further explore whether the chemical coagulation system was influenced by PEG-20k, which may have accounted for the hypocoagulative profile on TEG, we examined the activity of the intrinsic and extrinsic coagulation pathways by measuring the Prothrombin Time (PT) and activate Partial Thromboplastin Time (aPTT), respectively, in activated whole blood. In Figure 14, the graphs show that PEG-20k did not affect the PT times since both control and polymer treated blood had both normal and identical PT times. The same was observed for aPTT when the samples were activated by Kaolin. However, when the blood samples were activated using silica, which ius a common activator in clinical hematology labs, The PEG-20k diluted blood had an infinite aPTT time, suggesting that Peg-20k was completely blocking coagulation by the intrinsic pathway. Other data not shown also show a completely inactive factor VIII system with PEG-20k. In effect creating a chemical state of severe hemophilia. However, further experiments using kaolin activators show no effect of PEG-20k dilution on the intrinsic coagulation pathway. It seems that PEG-20k polymers are able to completely sequester or block the the actions of silica in initiating the intrinsic cascade, perhaps by passivation of the platelet surface to silica binding. Although this effect has been shown to be an artifact, it is important to document because laboratories ordered to test aPTT in trauma patients (or any patient) that is receiving solutions with PEG-20k must be advised to only use kaolin activators in their assays and NOT silica. Silica activation will result in artifactually high aPTT times and may lead to inappropriately treating a patient for a severe coagulopathy that he in fact does not have.

Figure 15

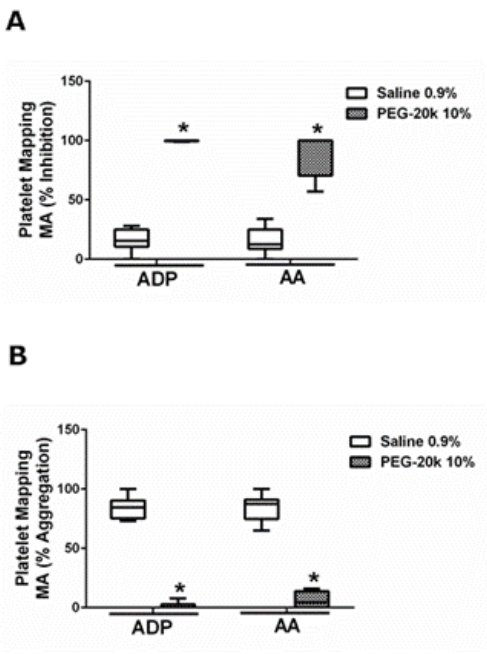


Thrombin generation by the system, from both platelet and chemical plasma components, is shown in Figure 15 for both PEG-20k and saline diluted platelet rich plasma (PRP) using low dose (1 pM) tissue factor as the trigger. We detected a very slight but significant decrease in most of the outcomes of the thrombin generation assay (CAT Assay, Calibrated Automated Thrombogram). It is not known if this is a scientific difference without a clinical effect or if there is a causal relationship between slightly lower thrombin release in the PEG-20k samples. Given the entirety of these data (including data discussed below), we believe the slightly lower thrombin generation with PEG may be more an effect of the PEG-20k on platelets rather than a cause of the hypocoagulative responses observed.

The platelet contribution to clot formation under specific platelet stimulation conditions with either ADP or arachidonic acid (AA) was conducted in a platelet mapping TEG study using blood diluted with saline (control) or PEG-20k. These results are shown in Figure 16. Platelet activation with both ADP and arachidonic acid induced a normal clot formation response on TEG as shown by the high aggregation response (Panel B) and low inhibition response (Panel A). However, dilution with 10% PEG-20k caused a significant inhibition of the ADP and AA response (Panel A) and a significant decrease in the ADP and AA-induced aggregation response (Panel B), relative to the saline control.

ADP activation of platelets in PRP induces a rapid expression of IIb / IIA protein complexes and P-Selectin that are detected by specific binding of antibodies to both (PAC1 and anti-CD62P, respectively). These data are

Figure 16



shown in Figure 17 for both saline and PEG-20k diluted PRP samples. While there were significant increases in both PAC1 (88.5%) and CD62P (59.7%) antibody binding to ADP-activated platelets, compared to the non-activated state with saline dilution, the effect was not different when PEG-20k was used as the diluent (87.4% increase for PAC1 and 62.5% increase for CD62P).

While the coagulation component of blood clot formation may not be a significant target of PEG-20k in clot formation at high doses, the platelet activation components may be a significant target. Platelet receptor expression (PAC1 and CD62P) after ADP activation was not altered by PEG-20k, but the functional effects of ADP activation on the platelet component of clot formation was, as seen with platelet mapping using stimulation with both ADP and arachidonic acid. This indicates that interference by PEG-20k in platelet clot formation may be downstream from IIb/IIIa receptor expression after activation. It is tempting to suggest, based on the available evidence to date, that PEG-20k may interfere with IIa/IIIb binding to fibrinogen, thereby interfering with platelet aggregation per se and the amplification of downstream receptor signaling by epinephrine, ADP, PAF, collagen, and thromboxanes on platelet aggregation. This is supported by the data showing the MA on platelet mapping and in regular TEG to be reduced with PEG-20k. Furthermore, the lower k and angle values seen with PEG-20k solutions, which mimic a functional state of hypofibrinogenemia in the presence of normal fibrinogen levels, may be due to blocking of the IIb/IIIa receptor and inhibition of fibrinogen binding and platelet aggregation. Therefore, PEG-20k may induce a state of chemical thrombasthenia at higher concentrations while not significantly affecting the chemical coagulation cascades. This is further supported, albeit indirectly, by data demonstrating robust effects of PEG-20k solutions on red blood cell sedimentation rates, which are competitively inhibited by smaller PEG polymers (Figure 7). The almost 20 fold increased sedimentation rate of RBCs seen with PEG-20k (Figure 13A) suggests avid cell membrane binding and cross linking to form denser packed cell particles. The inhibition of this effect with shorter chain polymers strengthens the concept of cells (RBCs) having fixed numbers PEG polymer binding sites that can be cross linked by larger polymers but competitively inhibited by short chain polymers of PEG (Figure 13B).

Figure 17

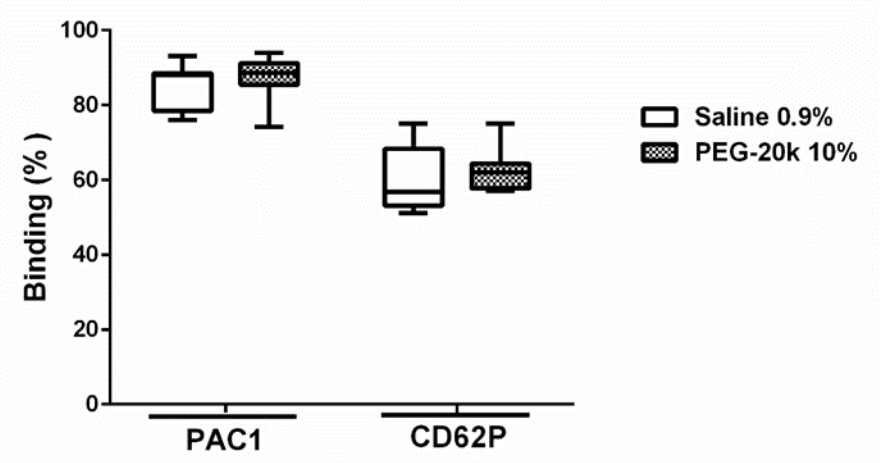
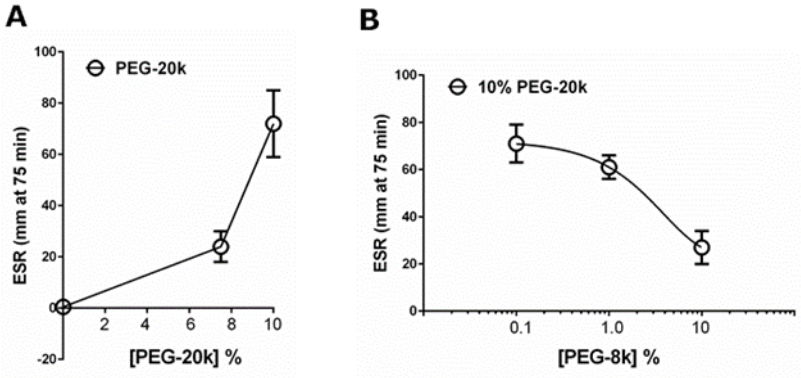


Figure 18

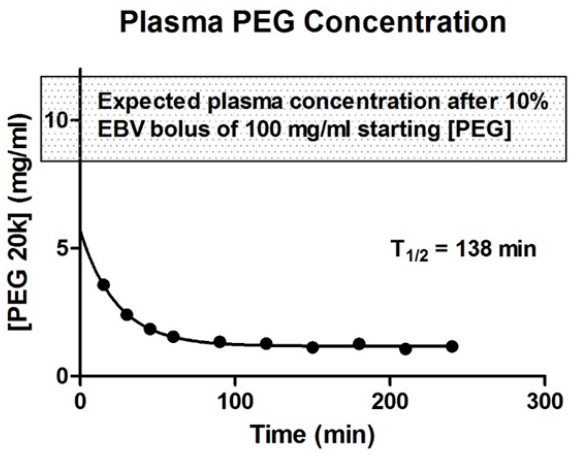


If this binding were to occur in platelets too, then some platelets may be functionally removed from binding with fibrin, fibrinogen, and adhesion molecules to alter the platelet component of clot formation, as documented clearly in these two studies. This proposed parallelism between PEG-20k interactions with RBCs and platelets has not been demonstrated empirically but such a nonspecific passivation effect seems reasonable to postulate from the very strong ESR effects of PEG-20k on red cells (Figure 18) and from the known affinity of PEG polymers with cell membrane components, including on platelets. Further studies using fluorescent or electron microscopy imaging may be useful to resolve what is happening to the platelet when PEG-20k is around under clot forming conditions.

In conclusion, this study has expanded our search for a mechanistic explanation for the identified effects of PEG-20k solutions on whole blood coagulation seen in healthy volunteers and trauma patients. We have learned that PEG-20k has little effects on the intrinsic and extrinsic coagulation pathways and on the availability of critical non-catalytic proteins such as fibrinogen and vWF. The effects of PEG-20k solutions on platelet activation may suggest that the predominant effect of these solutions on whole blood clotting at high concentrations may be due to interference with the normal platelet function during clot activation that mimic a state of mild functional thrombocytopenia, platelet passivation, or thrombasthenia.

Blood levels of PEG-20k in shocked pigs after acute resuscitation

Figure 19



We developed data in the hemorrhagic shock model in the pig showing the maximum peak blood levels after resuscitation. This is critically important if we are to interpret the ex-vivo coagulation data correctly. From the ex-vivo blood clotting studies we know that 10% dilution of a 10% PEG-20k solution causes a mild hypocoagulative state that we may need to worry about in trauma patients. Most of the studies in this quarter were directed at elucidating the mechanisms of action of this effect, which we believe we understand clearly now. However, this is all moot if the blood levels of PEG-20k are not high enough to cause these effects. From previous studies using the ex-vivo coagulation studies we also determined that slightly lower doses of PEG-20k DO NOT cause interference with coagulation by platelet mechanisms. A PEG-20 concentration of > 10 mg/ml causes coagulopathy whereas < 7.5% does not. So, we needed to determine the PEG concentrations at their highest level right after administration in a pre-clinical model of trauma and severe hemorrhagic shock. These blood levels are shown in Figure 19.

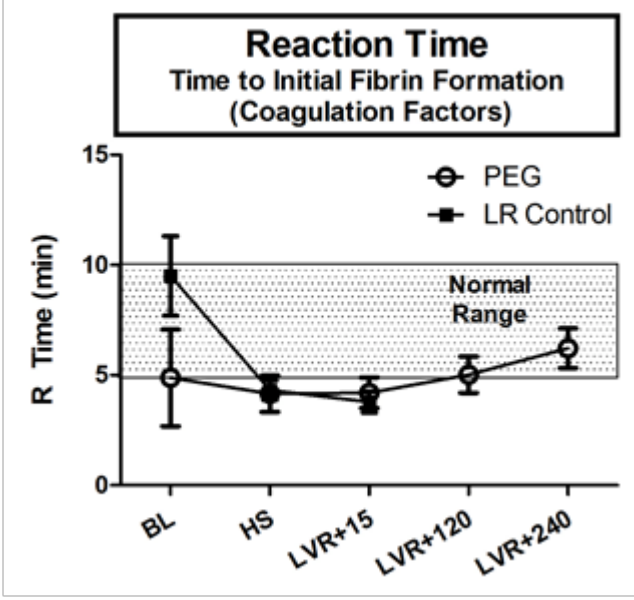
These data clearly show that the peak PEG-20k concentrations in the blood are at about 5 mg/ml, which is far lower than the 10% needed to cause coagulopathies. Therefore, the previously observed hypocoagulation seen after PEG-20k dilutions in whole human blood are dose dependent and likely will not apply to patients receiving low volume resuscitation with PEG-20k based crystalloids. While it is good to understand the

mechanisms involved in this effect (hypocoagulation), it remains moot since the highest achieved in-vivo dose is twice lower than the threshold dose needed to induce the effect.

Actions of PEG-20k based LVR solutions on coagulation and platelet function in pigs in-vivo after severe blood loss and low volume resuscitation.

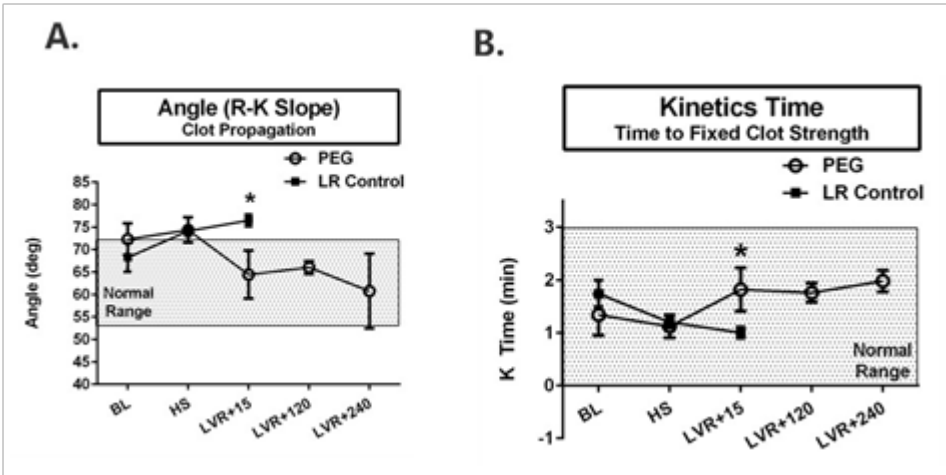
Methods and Model: Pigs (40kg) were anesthetized and shocked by bleeding the animals to an arterial pressure of 35 mm Hg until their oxygen debt achieved a set value, which was standardized to one of the three endpoints (determined empirically from the previous acute studies):
1. Plasma lactate reaches 6-7mM
2. Total blood loss reaches 53% of estimated blood volume
3. Total hemorrhagic shock time under the above conditions reaches 112 minutes
After the standardized shock period (oxygen debt) is reached, a low volume resuscitation is given by infusing IV over 10 minutes a volume of either a.) Lactated Ringers solution (volume control) or b.) A solution of 10% PEG-20k in lactated ringers, where both solutions are given at a volume equal to 10% of the estimated blood volume of the pig (about 280 ml). The pigs are allowed to survive under these conditions up to 240 minutes. At points during the experiment, blood samples are obtained for analysis by thromboelastography (TEG) using a Haemonetics 5000 TEG machine with kaolin activation of citrated whole blood (reversed with calcium chloride). Following TEG analysis, the major TEG parameters of R, k, angle, MA, and CI were reported for the two groups at various time points along the experimental time line.

Figure 20



The reaction time of the coagulation system (R) is the time to initial fibrin formation following kaolin activation and is an indicator of the coagulation factors present. Figure 20 shows that none of the R values were significantly outside of the normal range and were not different between the two groups at any time during the study. These values are an average of 5 independent TEG values from 5 pigs in each group. Only the animals assigned to the PEG-20k resuscitation group were able to report all values up to the 240 minutes past LVR because all of the LR treated volume controls died after the 15 minute sampling period but before the 120 minute period.

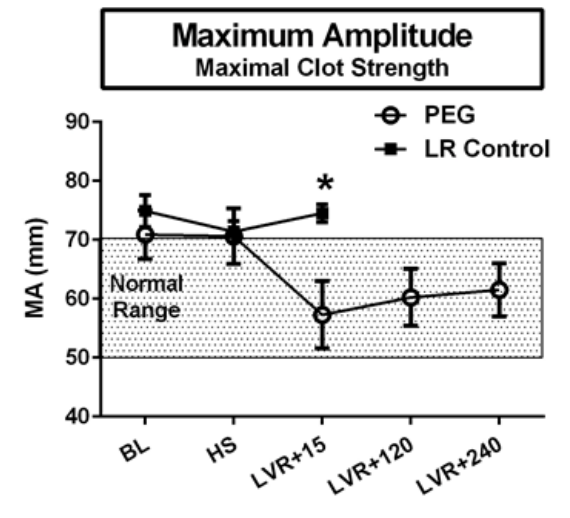
Figure 21



Other TEG values observed from the two groups of pigs include the angle and the k values, which are both indicative (albeit reciprocal) of the rate of clot propagation after it has started to form (the end of R). This value has been attributed to coagulation factors, fibrinogen levels, and minor platelet function. These values are shown in Figure 21, where panel A shows the "angle" values and panel B shows the "k" values. Although most of the values in either group were within the normal ranges, the values for the PEG-20k group were significantly different from the corresponding volume control group immediately after LVR was given (15 min), which is the time when PEG-20k plasma levels are at their peak. The angle was significantly higher and the k value was significantly lower in the PEG group. Since both k and angle are reciprocal values that indicate the rate of clot formation, we can conclude that a slightly hypercoagulable state exists in the LR treated trauma pigs early after shock and resuscitation, like it does in trauma patients, and PEG-20k resuscitation reverses and normalizes the propagation rate. After the initial infusion ends (15-60 min), this effect is lost.

The MA value on TEG followed a similar pattern as angle and k immediately after administration of PEG-20k LVR solutions in shocked pigs (Fig 22).

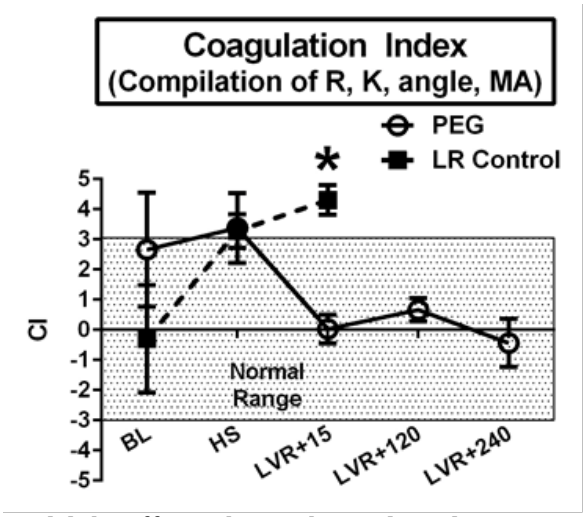
Figure 22



The MA is a measure of both platelet function (80%) and coagulation (20%) and represents the maximum clot size and clot strength. These data clearly show a slightly hypercoagulable state in the untreated volume controls after shock and resuscitation, relative to the pigs given an LVR solution containing 10% PEG-20k. Specifically, the clot size and stiffness was reduced by PEG immediately after LVR infusion when PEG-20k blood levels are at their maximum. Again, it could be argued that normalizing the well-recognized hypercoagulative state present in most patients immediately after the shock state, usually seen early as the patient enters the trauma system, would be beneficial for later perfusion of vital tissues as resuscitation continues.

Finally, the overall coagulation index (CI) is shown in Figure 23. The CI is a mathematical modeling of multiple TEG attributes including R, k, angle, and MA.

Figure 23



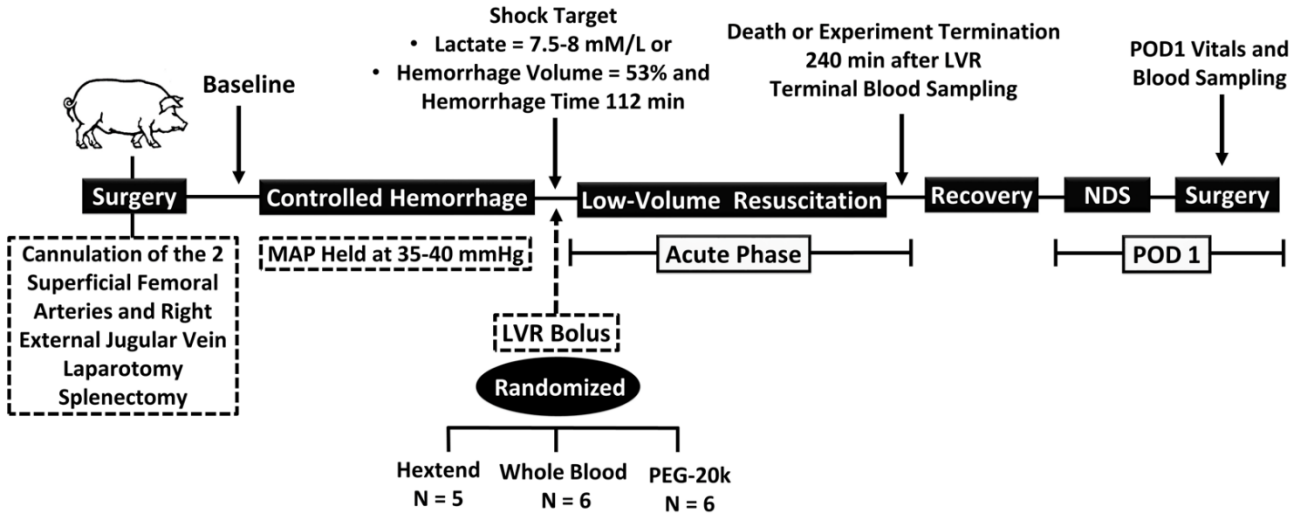
The CI for these pigs again confirms the temporary hypercoagulable state immediately after low volume resuscitation and the normalizing effect of the PEG-20k solutions. The true extent of the hypercoagulable state in this model is not known because all of the control pigs died after the 15 minute sampling period. However, in trauma patients it is believed to last about 1-2 hours after traumatic shock and to significantly contribute to perfusion problems later.

SUMMARY: PEG-20k LVR solutions do not produce a hypocoagulative state when used in severely injured traumatized and shocked pigs, unlike the human ex-vivo blood TEG data showing a hypocoagulative effect. This is because the blood levels of PEG-20k are 2-3 times lower in-vivo than the levels that we used previously in-vitro to model the effect. This is obviously welcome news since now we can enjoy the remarkable efficacy of these solutions on resuscitation and perfusion without having a bleeding or clotting problem. In fact, these data suggest that the PEG-20k solutions may reverse the often seen temporary hypercoagulative state precipitated by severe shock, which would serve to prevent microthrombi formation and improve further microcirculatory oxygen transfer to tissues after resuscitation.

Actions of PEG-20k based LVR solutions on survival in shocked pigs.

An important objective for the project was to conduct survival studies in the pig shock model to determine the longer term effects of PEG-20k LVR resuscitation on the pigs' cardiovascular function, mental and neurological function, and overall survival. The experimental design is depicted in Figure 24.

Figure 24



After 16-20 hours of fasting, 17 male Yorkshire pigs (Archer Farms, Darlington, MD) weighing 34.8 ± 3.1 kg were sedated with intramuscular ketamine (20 mg/kg) with xylazine (2 mg/kg) followed by anesthesia induction using intravenous propofol (2-3 mg/kg). Anesthesia was maintained with isoflurane at 1% to 2% in room air

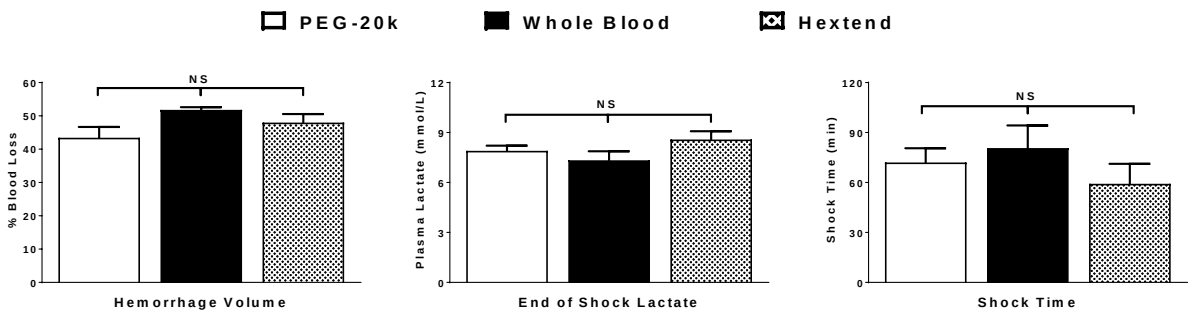
(FiO2 of 0.21) while on mechanical ventilation adjusted to an end tidal CO_2 of about 40 mm Hg. A circulating water-warming pad was used to maintain normothermia. Both superficial femoral arteries were cannulated for blood pressure and heart rate monitoring (PowerLab, ADInstruments inc., Dunedin, New Zealand) and for controlled arterial hemorrhage. Additionally, the external jugular vein was cannulated for fluid administration. A laparotomy and splenectomy were performed to produce soft tissue and organ injury and to mitigate the effect of autotransfusion of stored RBCs in the spleen.

Hemorrhagic Shock Model: An intravenous fluid loading bolus of LR solution (10 mL/kg) was administered over 10 minutes before baseline data and labs were obtained. Controlled hemorrhage was then initiated by arterial bleeding at a 2 ml/kg rate using a Masterflex® peristaltic roller pump (Cole-Parmer, Chicago, IL) until mean arterial pressure (MAP) reached 35-40 mmHg. After allowing the animal to compensate to a MAP of 45-50 mmHg, bleeding was resumed until one of the two shock endpoints was reached: 1.) A plasma lactate of 7.5-8.5 mmol/L was reached within 112 minutes of hemorrhage time and under a total hemorrhage volume of 53% (TBV) or 2.) Both hemorrhage time and hemorrhage volume limits were met without achieving the lactate goal.

Low-volume Resuscitation and Study Outcomes: Once the shock endpoint was reached, the animals were randomized to receive an intravenous bolus (over 5 minutes) equal to 10% TBV (LVR) of either of PEG-20k (n=6), WB (n=6), or Hextend (n=5). During the controlled hemorrhage, blood was stored in a Viaflex plastic bag containing sodium citrate and was used for resuscitation in the animals randomized to the WB group (autologous blood transfusion). Vital signs were recorded and lactate and hemoglobin levels were measured every 15 minutes after LVR. The experiment was terminated and the animals euthanized when MAP consistently dropped below 30 mmHg. If the pigs survived for 240 minutes after LVR, they were recovered from anesthesia. Surviving animals were weaned off anesthesia, recovered, provided post-operative analgesics (Buprenorphine SR), and allowed access to food and water. On postoperative day 1 (POD1), a 24-hour neurologic assessment score was determined and the pigs were taken back to the operating room for a terminal data and specimen recovery procedure. The primary outcomes of the study were the 24-hour survival rates and NDS. Secondary outcomes included: MAP, plasma lactate concentrations, and hemoglobin values. Neurologic function on POD1 was evaluated using a standardized scoring that considers behavior and level of consciousness, breathing pattern, cranial nerve function, and motor and sensory function. An NDS of 0–40 is considered as absence of neurologic deficit, a NDS of 400 as brain death. Finally, an indicator dilution method of hemoglobin was used to estimate changes in intravascular compartment volume after LVR assuming no further blood loss was allowed after reaching the controlled hemorrhage endpoint. Different hemodilution-based techniques have been used by others to calculate intravascular volumes in various clinical and experimental settings and we have previously validated the use of hematocrit changes against fluorescein isothiocyanate (FITC)-labeled albumin in a rat model of controlled HS.

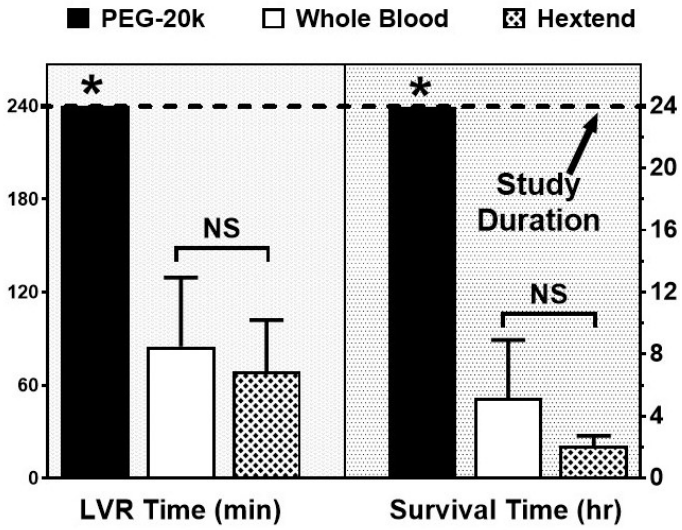
At baseline, animals in all groups had comparable weigh, MAP, lactate, hematocrit, creatinine and other measures of organ function. There also were no differences between groups in any of the shock parameters before the start of LVR (Figure 25)

Figure 25



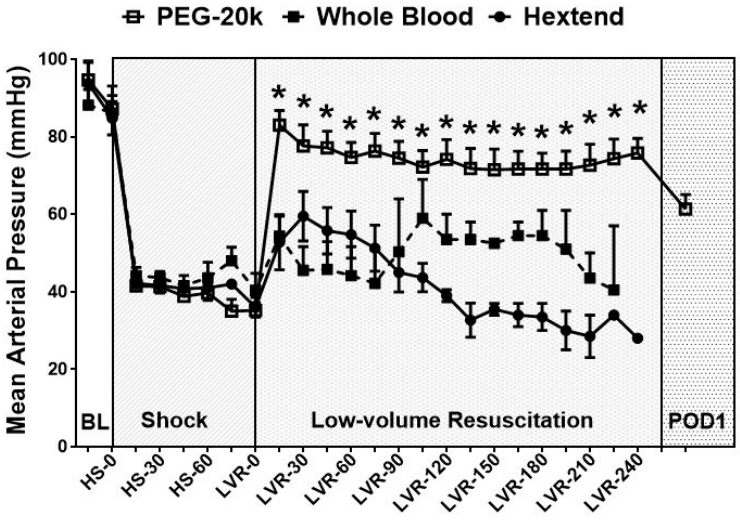
All animals (100%) in the PEG-20k group survived 24 hours compared to only one (16.7%) in the WB group and none (0%) in the Hextend group (p=0.001). Consequently, mean survival time was substantially longer in the PEG-20k group (24 hours) compared to the 2 other groups (Fig 26).

Figure 26



Following resuscitation, MAP increased to higher levels with PEG-20k compared to WB and Hextend ($p < 0.05$). These significant differences were observed throughout the acute phase of the experiment (LVR-15 to LVR-240). Again, no statistical differences were observed in blood pressure between the WB and Hextend groups (Figure 27).

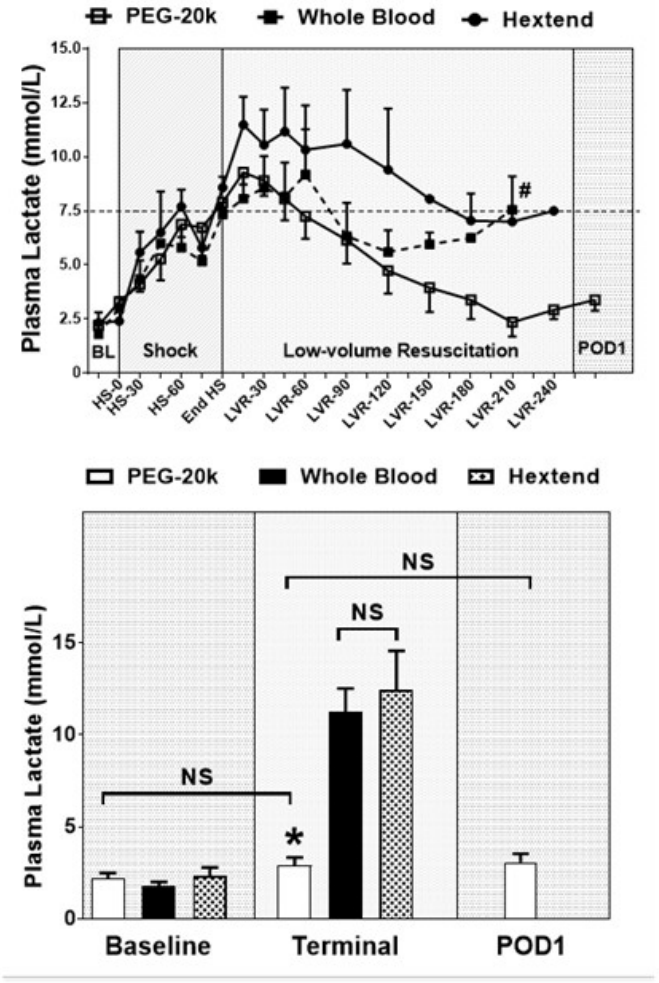
Figure 27



LVR with PEG-20k resulted in a complete lactate clearance by the LVR-240 time point.

Normal lactate was maintained for 24-hours despite a decrease in MAP from 75.8 ± 9.1 at LVR-240 to 61.3 ± 9.1 mmHg at POD1 ($p = 0.039$). Lactate at LVR-240 (terminal) was 2.9 ± 1 mmol/L which was not significantly different than baseline or POD1 levels. The one animal that survived 24 hours in the WB group had a terminal lactate level of 7.4 mmol/L. Lactate clearance, as well as MAP improvement, did not continue in the animals resuscitated with WB and Hextend beyond 1-2 hours and terminal

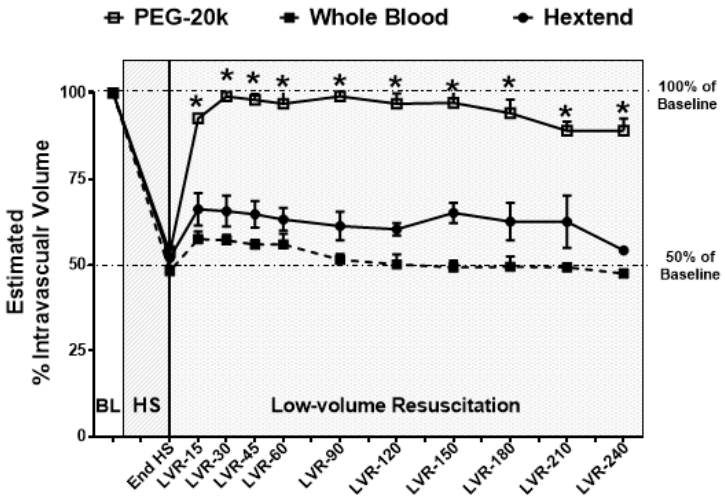
Figure 28



lactate was not different between the 2 groups (11.3 ± 3.1 and 12.5 ± 4.7, respectively) but significantly higher than the PEG-20k group (2.9 ± 1 mmol/L p = 0.001) (Figure 28).

Significant reductions in hemoglobin concentration were observed 15 minutes after LVR with PEG-20k and throughout the 24-hour study ($p < 0.01$). Hemoglobin decreased from 9.3 ± 0.7 g/dL at the end of shock to 5.9 ± 0.9 g/dL at LVR-240 ($p < 0.001$). POD1 hemoglobin was 6.5 ± 1.3 g/dL which was not significantly different than the levels measured at LVR-240. Intravascular volume, which similarly decreased in all groups to an average of 51.1% at the end of HS, was restored to 99% ± 4.6% of baseline volume 30 minutes after LVR with PEG-20k. This volume expansion was maintained for 3.5 additional hours. On the other hand, post-LVR intravascular volume peaked 15 minutes after LVR with WB and Hextend to 57.5 ± 5.5% and 66.2 ± 10.5%, respectively. These increases were not statistically different between the 2 groups but significantly lower than the PEG-20k group ($p \leq 0.001$). The small volume expansion with whole blood or hextend was lost in about 2 hours after administration. (Figure 29).

Figure 29



Uncontrolled hemorrhagic shock in rodents

An important objective for the project was to determine the performance of a PEG-20k LVR solution in a more clinically relevant model of hemorrhagic shock that is uncontrolled. Specifically, the patient is actively bleeding at the time of resuscitation for a reasonable period of time that models the time required to stop all bleeding (usually in a surgical facility). The prior controlled hemorrhage models are useful for extremity bleeding where blood loss can be definitively stopped by a tourniquet. In pre-hospital battlefield conditions involving polytrauma, many injuries are a mixture of both so it is important to model bleeding under uncontrolled conditions to determine how well the new polymer solutions perform. To that end, we report both the model and the first 6 studies, which provide insights as to the trajectory of the data.

Adult male Sprague-Dawley rats were anesthetized with isofluorane. A polyethylene catheter was placed in the femoral artery for blood pressure monitoring and blood sampling and another catheter was placed in the contralateral femoral vein for administration of fluids. An arterial blood gas sample was then obtained to measure baseline lactate. A 3 cm laparotomy incision was made and the stomach was gently pulled to the right side of the abdomen to allow visualization of the spleen. The spleen was kept inside the abdomen and attention was made to avoid stretching the vessels around the spleen and stomach and compromising the blood supply to the spleen.

The spleen was then gently lifted using a Q-tip to allow visualization of splenic artery branches under the spleen. Two transverse cuts were made using sharp operating scissors in 2 locations in the middle of the spleen between branches of the splenic artery going into the spleen while avoiding injury to the arteries (Figure 30, left). This was immediately followed by a tail cut of 75-80% of the total length of the tail. Blood from the tail was collected in a pre-weighted tube taped to the edge of the surgery table (Figure 30, middle). The spleen was allowed to bleed freely into the abdominal cavity and the abdominal wall was closure with a running suture. An umbilical tape was temporarily tied loosely around the tail to stop the bleeding when MAP decreased to a level lower than 30 mmHg (Figure 30, right).

When lactate reached 7.5-9 mmol/L, the tail was clamped and low-volume resuscitation was initiated with either 10% PEG-20k (treatment group), Hetastarch (Polymer control) or lactated Ringer's solution (volume control). LVR with a volume equal to 10% of the rat's total blood volume was given over 5 minutes. MAP was recorded and serial lactate measurements were obtained after LVR was started. LVR time and survival times were measured at the end of the experiment. LVR time was defined as the time after LVR when lactate reached 7.5 mmol or more excluding the initial peak (mostly at LVR-15 time-point), as long as it was followed by a decrease in lactate to a level lower than that observed at the end of the hemorrhagic shock (before LVR).
At the end of the experiment, the laparotomy incision was reopened and the free peritoneal blood (in addition to large clots if applicable) were collected on pre-weighted gauze. Percentage of total blood loss was calculated by adding the tail bleeding volume to the spleen bleeding volume and dividing it by the estimated total blood volume (calculated as the weight X 0.06 + 0.77 ml).

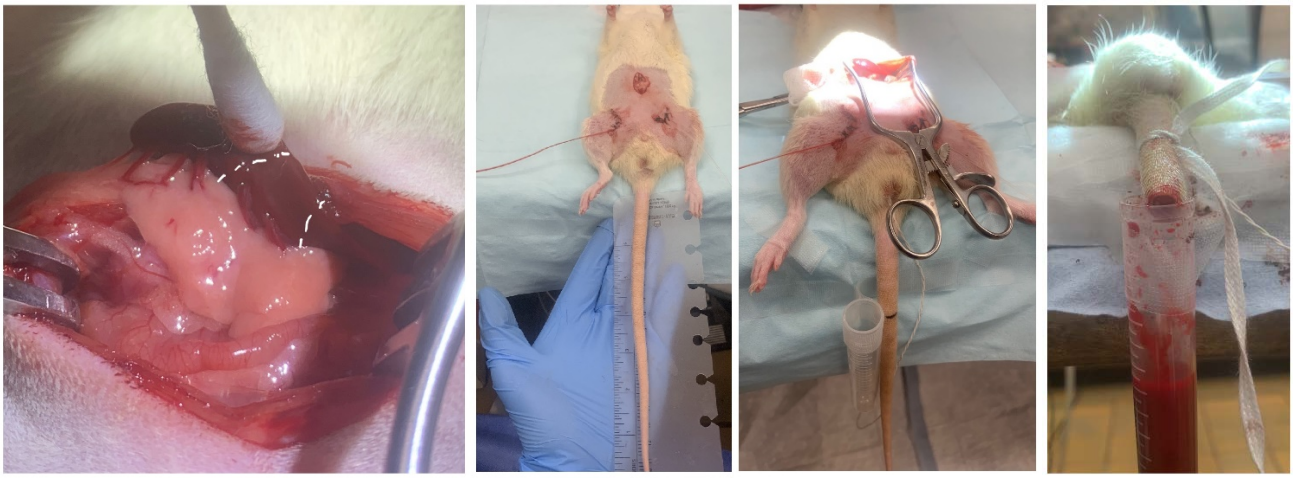
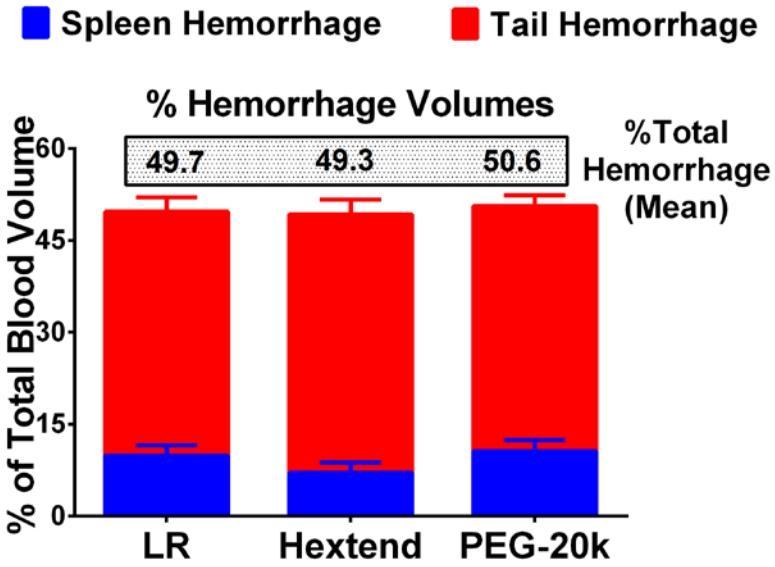


Figure 30. Uncontrolled hemorrhage model of combined tail cut (right and middle) and splenic parenchymal transection (left)

Three groups of rats were used as determined by what IV solution was used for ther low volume resuscitation. We used LR as a civilian volume control, Hextend as a military LVR, and PEG-20k solution as the experimental group. All LVR volumes were the same (10% of the estimated blood volume). The bleed volumes (from spleen, tail, and total) for the 3 groups are shown in Figure 31.

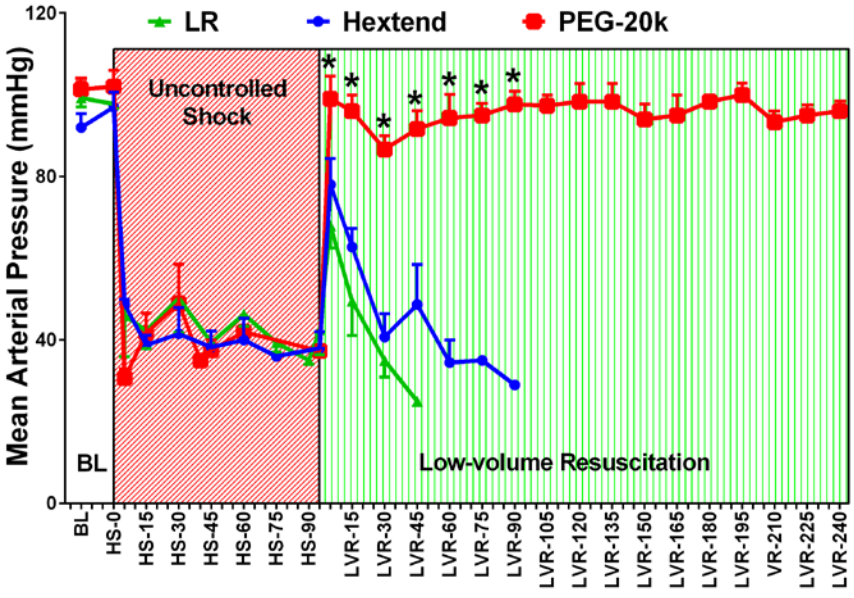
Figure 31



The bleed volumes between the groups were all very similar. The total bleed volumes were about 50% in all groups. This was a bit surprising since it is commonly believed that a higher perfusion pressure will "pop the clot" to restart bleeding that has not been controlled by tamponade or tourniquets. Although this inevitably happened, the effect of PEG-20k on this rebleed phenomenon may not be as bad as originally thought.

In this model, we observed a striking benefit of the PEG-20k fluid compared to the LR volume control. Figure 32 shows the effects on mean arterial pressure (MAP) over time in the three groups resuscitated with LR, Hextend, or PEG-20k.

Figure 32

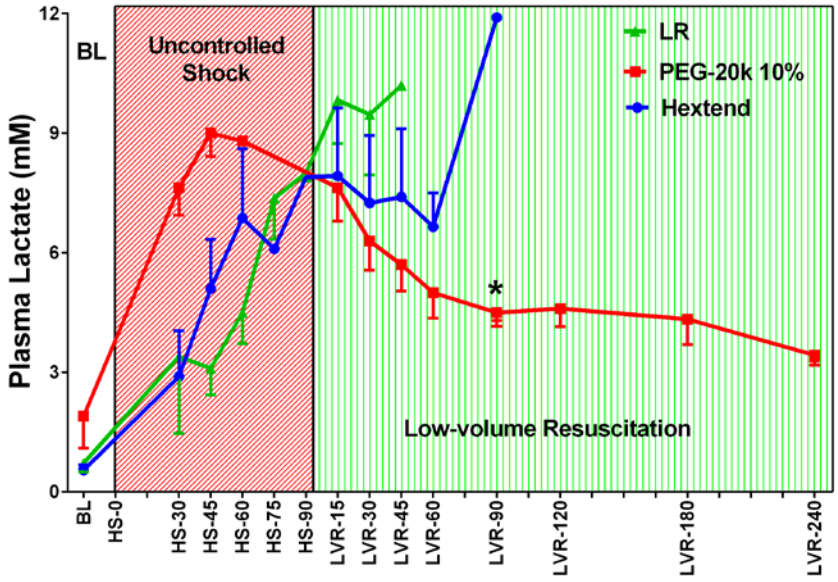


Clearly, rats resuscitated with the PEG-20k solutions had a much safer arterial pressure during the resuscitation period and the survival was almost 3 times longer..

A similar story is told when the outcome is the plasma lactate concentrations during resuscitation (Figure 33). As typically occurs in the saline resuscitated controls, the lactate continues to climb and the rat dies shortly after LVR administration. This is mainly because the small volume is rapidly third spaced and contributes almost nothing to oxygen delivery.

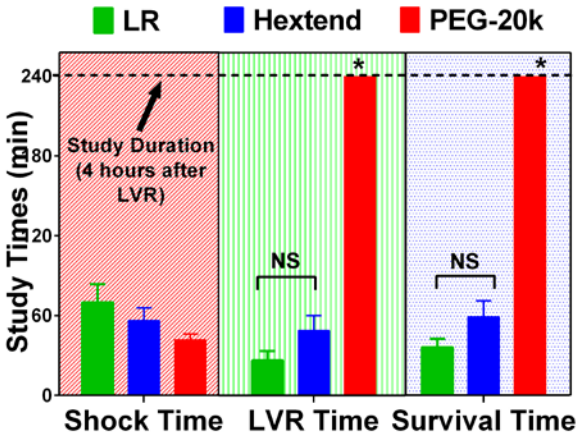
However, an equivalent volume of PEG-20k produces increased perfusion and serves to repay debt (even while still actively bleeding). This actually drives down lactate during LVR as debt is paid back.

Figure 33



Finally, the group comparisons in the uncontrolled hemorrhagic shock model can be measured by outcomes involving both LVR time and total survival time, which in this study are the same (**Figure 34**). Specifically, the LVR time, which measures the tolerance of the subject to the low volume state, was prolonged to 4 hrs or a 5-6 fold increase over the LR and the Hextend groups. The same is seen for total survival time. It needs to be emphasized that these survival times in the PEG-20k group is an underestimation of the true times because the animals were euthanized after 4 hrs, which was the total study duration times. They clearly would have survived longer had we allowed them to do so.

Figure 34



Summary

1. Low volume resuscitation with solutions of PEG-20k (10% w/v) produce geometrically better results in acute short-term lethal hemorrhagic shock studies compared to civilian controls (LR solution or whole blood) and military controls (Hextend).
2. Survival times and tolerance to the low volume state dramatically increase after PEG-20k resuscitation. Secondary outcomes also improve since oxygen debt is paid back in 4 hrs after resuscitation resulting in normalization of plasma lactate, base excess, and central blood pressure, relative to comparable volume controls (LR, Hextend, whole blood).
3. PEG-20k solutions at 10 mg/ml in ex-vivo whole blood from either normal volunteers or acutely injured trauma patients produce a short lived mild coagulopathy characterized as a mild thrombasthenia and minor reduction of factor XIII-induced fibrin cross linking activity.
4. This may be due to interference by PEG-20k polymers with platelet cross linking to fibrinogen via interference with the platelet IIb/IIIa receptor / fibrinogen binding in ADP and collagen activated platelets.
5. Peak plasma concentrations of PEG-20k after IV resuscitation in lethal shock and LVR studies in pigs are 3 fold lower than previously estimated (3 Vs 10 mg/ml). This reduces in magnitude and duration of the mild coagulopathy seen on thromboelastography.
6. The half-life of IV PEG-20k is about 2 hours and excretion is almost exclusively via renal clearance.
7. Intravascular volume is rapidly returned to control baseline values within 30 minutes after administration of PEG-20k (10% solution administered at 10% estimated blood volume) in pigs with an acute 50% blood loss. This causes significant hemodilution and reduces hemoglobin concentrations. In spite of this marked hemodilution, plasma lactate values fall rapidly after PEG-20k LVR to baseline indicating oxygen debt is repayed after resuscitation. Whole blood or Hextend does not change the intravascular volume and oxygen debt is not repayed (as indexed by rising lactates).
8. In lethal hemorrhage in pigs, PEG-20k resuscitation leads to 24 hour survival with normal cardiovascular and metabolic outcomes and normal neurological behavioral scores. Whole blood or Hextend control groups die within 2 hours after resuscitation and do not survive.
9. Small volumes of PEG-20k lead to universal 24-hour survival in lethal shock without need for further blood components compared to whole blood controls where all pigs die within an average of 2 hours after resuscitation.
10. The use of PEG-20k crystalloids in low volumes needs to be strongly considered as an effective strategy for dramatically prolonging the safe field and transport time in severely shocked patients and for use as a blood free or blood product reducing strategy for hospital trauma resuscitation and mass transfusion protocols.
11. Low volume resuscitation with PEG-20k solutions in rats is significantly better than volume controls with LR solution in a model of mixed controlled and uncontrolled hemorrhagic shock that models polytrauma in the field.

What opportunities for training and professional development has the project provided?
This project has provided valuable training to (3) general surgery residents that take two years for research at the end of their second clinical year. Additionally, 2 post-doctoral fellows and one PhD student have been trained under this project at some level. One junior clinical faculty and one trauma division chief have also expanded their professional development under this project by presentations and attendance at national meetings where work from the project has been presented.

How were the results disseminated to communities of interest?
Through full length and abstracted publications in refereed journals, oral and poster presentations at national and local meetings, and through educational talks, conferences, grand rounds, and other venues at VCU.

4. Impact

The impact on the principal disciplines of the project are huge. Specifically, we describe a novel mechanism of resuscitation injury from metabolic cell and tissue swelling during circulatory shock, that when mitigated by the novel reagents developed in the project (PEG polymer based LVR solutions), lead to geometrically better outcomes compared to current state of the art IV resuscitation treatments. As a corollary, a significant realignment of our thinking is occurring about what root mechanisms of resuscitation injury are truly important and how unimportant contemporary thinking on the matter today really is. This cannot be ignored in light of the magnitude of the biological responses described in this project and the now evident mechanisms of action as described by this project and the one preceding it.

The impact of the project on technology transfer has been equally as large as the impact on the field of shock and trauma resuscitation and the underlying biology of reperfusion injury in shock. Specifically, the demonstration of long term (>24 hr) survival from lethal hemorrhage with the developed PEG-20k IV solution is driving commercialization attempts. This is especially true when we compared the PEG-20k solution to what is currently recognized as the gold standard in clinical resuscitation (blood). The last project and the manuscript submitted to Annals of Surgery (appended) has lit a fuse in the clinical trauma surgery field. Presentations at national trauma meetings are literally packing the auditoriums as interested stake holders in the filed want to hear more about these pre-clinical responses, especially when compared to blood and blood products. Recently, I recruited a commercialization partner from Northern Virginia to help me commercialize this product as quickly as possible. After considerable due diligence, Gerard Eldering (CEO of InnovateTech), has agreed to help the PI form a company and provide company leadership to attract investment capital and move the product through FDA trials as a new medical molecular device for approval and clinical testing. The claim and label will be to dramatically restore tissue perfusion. The goal is to continue de-risking and testing with investor resources and grants until a larger established company takes enough of an interest to either purchase the licensing agreement or the entire company, which is developed solely to commercialize PEG-20k for use in low flow states including shock and trauma. Although we have identified countless uses for this solution including ICU care, hospital based resuscitation, bloodless transfusion, CPR, cardiopulmonary bypass, burn resuscitation, compartment syndrome, organ donation, neurotrauma, mass transfusion, critical illness, etc, we have sufficiently de-risked the product in pre-hospital resuscitation. Now we can comfortably go to FDA to label this for use as a product that can significantly increase tissue perfusion under low flow states. We still need much more experimentation in all of these other uses but the work in shock may be at a point now where commercialization becomes a self-sustaining force.

The impact on society is large. This concept, and its associated predicate product, works under a new paradigm that produces results orders of magnitude more effective than anything used in the last 120 years. Furthermore, the realignment of the underlying mechanisms of injury in low flow states opens up a huge list of uses for similar medical conditions that share the same underlying mechanisms of injury. Therefore, there is a very large potential impact on medical care from field to hospital that can be improved. For example, the ability of this product to substitute for whole blood or blood products and the potential for use with blood products to both improve outcomes and stretch limited resources is highly impactful in society. The resources of the Red Cross for example could be greatly expanded by introducing these polymer solutions into current resuscitation or mass transfusion protocols. The ability of these solutions to substitute for whole blood or oxygen carriers in the filed because of their abilities to greatly increase the efficiency of oxygen transfer in the microcirculation will revolutionize prehospital and field transport medicine for traumatic injuries. This material is geometrically more effective than blood and requires no special handling. It is stabile under harsh conditions for very long times and is inexpensive to make. The impacts to medicine and society are only limited to our innovative thinking about potential uses.

5. Changes / Problems

There have been no changes to the project or any significant problems that weren't overcome.

6. Products

Full length publications

Plant V., Parrish D., Lindell S, Limkemann A, Reichstetter H, Ferrada P, Aboutanos M., and **Mangino MJ.**
Low volume resuscitation in hemorrhagic shock: Understanding the mechanisms of PEG-20k
J. Pharm. Exp. Ther. 361 (2): 334-340, 2017

Mangino, MJ, Liebrecht, L, Plant, V., Limkemann, A.
Crystalloid and colloid resuscitation-Hypertonic saline, starches, polymers, gelatins.
In: Hemorrhagic Shock: Recognition, Pathophysiology and Management
Nova Science Publishers, Chapter 8
Jose Pascual and Jeremy Cannon, Editors
2016

Liebrecht, L., Newton, J., Wickramaratne, N., Jayaraman, S., Han, J., Aboutanos, M., and **Mangino, M.J**.
Thromboelastographic Analysis of Novel Polyethylene Glycol Based Low Volume Resuscitation Solutions
PLoS One Nov 15;13(11):e0207147. doi: 10.1371/journal.pone.0207147. eCollection, 2018.

Liebrecht, L., Newton, J., Martin, E., Brophy, D., Wickramaratne, N., Jayaraman, S., Han, J., Aboutanos, M., and **Mangino, M.J**.
Effects of a novel low volume resuscitation solutions on coagulation and platelet function
PLoS ONE May 14(5): e0215386 https://doi.org/ 10.1371/journal.pone.0215386, 2019

Wickramaratne, N, Kenning, K., Reichstetter, H., Blocher, C., Li, R., Aboutanos, M., and **Mangino, M.J.**
Acute Resuscitation with Polyethylene Glycol 20k: A thromboelastographic Analysis
J. Trauma 87 (2): 322-330, 2019

Khoraki, J, Wickramaratne, N, Kang, HS, Xu, H, Archambault, C, Blocher, C, Li, R, Leichtle, S, Aboutanos, M, **Mangino MJ**
Superior Survival Outcomes of Polyethylene Glycol-20k Resuscitation Solution in a Preclinical Porcine Model of Lethal Hemorrhagic Shock
Annals of Surgery (Submitted)

Presentations at National Meeting

40th Annual SHOCK Conference, 2017, Fort Lauderdale, FL
Ex-vivo Coagulation Analysis of Polyethylene Glycol 20,000 (PEG-20k) Resuscitation Solutions using Thrombelastography
Loren Liebrecht

40th Annual SHOCK Conference, 2017, Fort Lauderdale, FL
Microcirculatory Effects of Polyethylene Glycol 20,000 (PEG 20k) During Resuscitation of Hemorrhagic Shock
Niluka Wickramaratne

Military Health System Research Symposium, 2017, Orlando FL
Microcirculatory Mechanisms of Polyethylene Glycol 20k During Resuscitation of Hemorrhagic Shock
Niluka Wickramaratne

77thg Annual American Association for Surgery of Trauma, 2018, Chicago, IL
Acute Resuscitation with Polyethylene Glycol-20k: A Thromboelastographic Analysis
Niluka Wickramaratne

Eastern Association for the Surgery of Trauma 2017
EFFECTS OF A NOVEL POLYETHYLENE GLYCOL 20K BASED RESUSCITATION SOLUTION
ON EX-VIVO COAGULATION AND PLATELET FUNCTION.
Niluka Wickramaratne

XIV Wolff Creek Conference, 2017, Richmond, VA
The Role of Cell and Tissue Swelling in Hemorrhagic Shock: New Opportunities for Low Volume Resuscitation and Tolerance Induction to the Low Volume State Using Cell
Martin Mangino

American College of Surgeons, 2017, San Francisco, CA
Polyethylene Glycol 20,000: A Novel Resuscitative Fluid for Hemorrhagic Shock
Niluka Wickramaratne

XV Wolff Creek Conference, 2019, Richmond, VA
Update on PEG-20k based LVR solutions in severe hemorrhagic shock: Survival, coagulation, and platelet function studies
Martin Mangino

American College of Surgeons 2019, San Francisco, CA

Low Volume Resuscitation with Polyethylene Glycol-20k Confers Survival after Severe Hemorrhagic Shock
Niluka Wickramaratne

Military Health System Research Symposium, 2019, Orlando FL
Low Volume Resuscitation with Polyethylene Glycol-20kfor Uncontrolled Severe Hemorrhagic Shock
Jad Khoraki

Military Health System Research Symposium, 2019, Orlando FL
Polyethylene Glycol 20k is superior to Hextend and whole blood for low volume resuscitation of hemorrhagic shock and soft tissue injury: 24 hour survival with normal neurological assessment
Jad Khoraki

American College of Surgeons Committee on Trauma 98th annual meeting, 2019, Richmond, Baltimore, Chicago
Low Volume Resuscitation with Polyethylene Glycol-20k Confers Survival after Severe Hemorrhagic Shock
Niluka Wickramaratne
Dr. Wickramaratne took the local and regional competition first place in Basic Science and she now heads to the national competition in Chicago for this study

Patents

Organ protection solutions and method of use
Patent number: 10300029
Abstract: An organ protectant solution which is intravenously administered includes at least one oncotic agent and optionally a high concentration of cell-impermeant molecules. Together, they promote transfer of water from cells to interstitium and into the capillaries, thereby preventing or reducing cell swelling, maintaining blood circulation and oxygenation of tissues, and extending the "Golden Hour" for traumatic and/or hemorrhagic shock patients. In addition, compositions comprising PEG-20k and methods of their use for preserving and/or reanimating harvested organs ex vivo for lengthy periods of time (e.g. at least about 8-24 hours) are also provided.
Type: Grant
Filed: October 14, 2016
Date of Patent: May 28, 2019

Assignee: Virginia Commonwealth University
Inventor: Martin Mangino

7. Participants and Other Collaborating Organizations

All VCU Contributors

Name: Niluka Wickramaratne, MD
Project Role: Post-Doc
Researcher Identifier:
Nearest person month worked: 12
Contribution to Project: Dr. Wickramaratne was the lead investigator and performed the shock studies (surgery, data collection, animal care)

Name: Loren Liebrecht, MD
Project Role: Post-Doc
Researcher Identifier:
Nearest person month worked: 12
Contribution to Project: Dr. Liebrecht is working on the patient platelet studies and helping with rat shock experiments

Name: Heather Reichstetter, LVT
Project Role: Technician
Researcher Identifier:
Nearest person month worked: 10
Contribution to Project: Heather helps Dr. Wickramaratne with the studies and with lab ordering.

Name: Caitlin Archambault, LVT
Project Role: Technician
Researcher Identifier:
Nearest person month worked: 6
Contribution to Project: Caitlin is the labs new lab manager and vet tech. She helps Dr. Wickramaratne with the studies and with lab ordering.
Name: Ru Li, MD, PhD
Project Role: Senior Scientist
Researcher Identifier:
Nearest person month worked: 6
Contribution to Project: Dr. Li is working on the pig and rodent studies. He is a new employee to the lab and is learning all aspects of this project**.**

Name: Jin Han BSN
Project Role: Research Nurse
Researcher Identifier:
Nearest person month worked: 1.2
Contribution to Project: Mrs. Han is assisting with the patient studies by maintaining the IRB protocol, retrieving samples, and consenting patients and volunteers.

Name: Martin Mangino, PhD
Project Role: PI
Researcher Identifier:
Nearest person month worked: 4

Contribution to Project: Dr. Mangino is supervising all of the studies, analyzing data, assisting with animal surgeries and with ex-vivo TEG studies, and is preparing statistical analysis, reports, and manuscripts.

Name: Charles Blocher
Project Role: Research Specialist
Research Identifier:
Nearest Person months worked: 3
Contributions to the Project: Chuck is a specialist in porcine studies, surgery, and anesthesia and is helping the lab start the pig shock studies, which are labor intensive and require specialized skills related to porcine anesthesia.

Name: Jad Khoraki, MD
Project Role: Post-Doctoral Fellow
Research Identifier:
Nearest Person months worked: 6
Contributions to the Project: Dr. Khoraki is helping with the pig studies and is conducting uncontrolled hemorrhagic shock studies in newly developed rodent models of uncontrolled hemorrhagic

8. Special Reporting Requirements:

None

9. Appendix

Seven full length papers attached

Appendix

1521-0103/361/2/334–340$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

https://doi.org/10.1124/jpet.116.239822
J Pharmacol Exp Ther 361:334–340, May 2017

Low-Volume Resuscitation for Hemorrhagic Shock: Understanding the Mechanism of PEG-20k

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Organ Preservation Laboratory, Department of Surgery, Division of Acute Care Surgical Services (V.P., D.W.P., P.F., M.A., M.J.M.), Department of Physiology and Biophysics (M.J.M.), and Department of Emergency Medicine (M.J.M.), Virginia Commonwealth University, School of Medicine, Richmond, Virginia

Received December 28, 2016; accepted March 6, 2017

ABSTRACT

Hemorrhagic shock leads to cell and tissue swelling and no reflow from compressed capillaries. Cell impermeants, including polyethylene glycol-20,000 (PEG-20k), reverse ischemia-induced cell swelling, extend low-volume resuscitation (LVR) time after shock, and increase tolerance to the low-volume state. The purpose of this study was to explore the mechanisms of action of PEG-20k containing LVR solutions. We hypothesized that PEG-20k acts as both an oncotic agent and an impermeant in the microcirculation, which moves water out of the space and into the capillaries to affect peripheral capillary filling and enhanced perfusion during the low-volume state. Rats were hemorrhaged until arterial lactate reached 9–10 mM/liter. Then, saline-based LVR solutions containing various impermeant materials were administered (10% blood volume). The LVR times for these solutions were determined by measuring the amount of time required for plasma lactate to climb back to 9 to 10 mM after LVR administration (low-volume tolerance). Capillary blood flow was measured by colored microspheres, and blood volume was measured by fluorescein isothiocyanate–labeled albumin dilution. Gluconate (impermeant), albumin (colloid), and PEG-20k (hybrid) increased LVR time over saline by 4-, 3-, and 8-fold, respectively. The combination of impermeant + albumin produced a biologic effect that was similar to PEG-20k alone. Capillary blood flow and plasma volume were decreased after shock with saline LVR but increased with PEG-20k, relative to saline. These data are consistent with the hypothesis that PEG-20k may act by establishing multiple osmotic gradients in the microcirculation to drive cell-to-capillary water transfer during hypovolemic shock.

Introduction

Minimizing the use of crystalloids and using blood products after trauma are now becoming mainstream in civilian trauma centers. Damage-control resuscitation is also emerging as the standard of care for the US Department of Defense, according to the Joint Theater Trauma Systems Clinical Practice Guidelines. When blood products are not available for resuscitation, crystalloid solutions are administered; however, only a fraction of infused crystalloid volume stays in the intravascular space, and the use of low-volume crystalloids has minimal effects on pressure and perfusion (van Lambalgen et al., 1990; Parrish et al., 2015a). The movement of crystalloid fluid from capillary to interstitium is compounded by the increase in capillary permeability from trauma-related inflammation and trauma-induced capillary leak syndrome (TICS) (Stein and Scalea, 2012). Furthermore, crystalloid resuscitation exacerbate TICS, acidosis, hypothermia, and coagulopathy (Duchesne et al., 2010; Stein and Scalea, 2012). Other resuscitation solutions, such as hypertonic saline or starch, have had disappointing results (Riha et al., 2011, 2013), including concerns and risks associated with their use (Cotton et al., 2006; Duchesne et al., 2010). There remains a need for a better crystalloid fluid that can be given at a low volume to resuscitate patients in hemorrhagic shock awaiting definitive treatment, especially for the prehospital setting. This study tested possible mechanisms of such a solution.

The dominant mechanism of injury in hemorrhagic shock is energy failure secondary to a lack of end-organ perfusion and loss of adequate microvascular oxygen transport with subsequent loss of aerobically produced ATP (Chaudry et al., 1974). As cells lose ATP owing to ischemia, the sodium pump shuts off and sodium ions enter the cell and accumulate as they run down their electrochemical gradient. Chloride follows electrogenically, and water enters the cell osmotically. As water enters ischemic cells, they swell and compress nearby vascular structures, which further aggravates ischemia by reducing local microcirculatory flow (Reffelmann and Kloner, 2002; Rezkalla and Kloner, 2002; Kloner, 2011). Swollen vascular endothelial cells and parenchymal cells compress capillaries to cause no-reflow, promote resuscitation injury, and limit oxygen delivery during the low-flow state and after

This work was supported by grants from the Department of Defense (W81XWH-12-1-0599) and (W81XWH-16-2-0040) to Dr. Mangino.
This study was presented, in part, at the 74th Annual Meeting of the American Association for the Surgery of Trauma, September 9–12, 2015, in Las Vegas, Nevada.
https://doi.org/10.1124/jpet.116.239822.

ABBREVIATIONS: FITC, fluorescein isothiocyanate; IL-1β, interleukin-β, LVR, low-volume resuscitation; PEG-20k, polyethylene glycol-20,000k; TICS, trauma-induced capillary leak syndrome.

full resuscitation (Reffelmann and Kloner, 2002). Reversal of cell swelling with cell impermeants has been used successfully in organ preservation for transplantation (Southard and Belzer, 1980) and in shock (Mees et al., 1982; Parrish et al., 2015a,b). These molecules are permeable to the capillary but impermeable to the parenchymal cell attributable to size and charge, thus creating an extracellular osmotic gradient that inhibits water entry into the cell.

Parrish et al. (2015a) have demonstrated reduced ischemia-induced cell swelling, increased tolerance to the low-volume state, and higher survival rates with administration of cell impermeant-based low-volume resuscitation (LVR) solutions in a rodent model of severe hemorrhagic shock. It was reasoned that if this occurs because of the creation of an osmotic gradient for fluid movement during ischemia, then a second gradient created with the addition of an oncotic agent in the resuscitation solution would augment the response. Indeed, when gluconate (a cell impermeant) was combined with polyethylene glycol 20,000 (PEG-20k, a colloid) in a LVR crystalloid solution, a marked potentiation in low-volume tolerance and blood pressure was observed (Parrish et al., 2015a). Surprisingly, when PEG-20k was used alone, it was equally effective as PEG-20k with the impermeant (gluconate). Additional studies demonstrated that PEG-20k, originally believed to be an oncotic agent, has both oncotic and impermeant effects because some of the material escapes the capillary space (impermeant effects) (Parrish et al., 2015b). This rather rare molecular behavior may explain how PEG-20k alone increased the LVR time (tolerance to the low volume state) 8-fold compared with either saline, mixtures of cell impermeants alone or pure oncotic agents alone (albumin). Specifically, this one agent may be doing double duty as both an oncotic and an impermeant molecule to generate a double gradient for fluid movement in the microcirculation. Therefore, we hypothesized that PEG-20k in shock acts via biophysical effects on water movement in the microcirculation through both cell impermeant and oncotic properties. These properties prevent cell swelling during ischemia, reload the capillaries with isotonic fluid from the interstitial space, and decompress the microcirculation, which all leads to increased capillary perfusion and oxygen transfer in the low-volume state. Figure 1 shows the hypothesized biophysical mechanisms of PEG-20k–based LVR solutions in low-flow and shock states. Here we present the results of experiments that support this hypothesis.

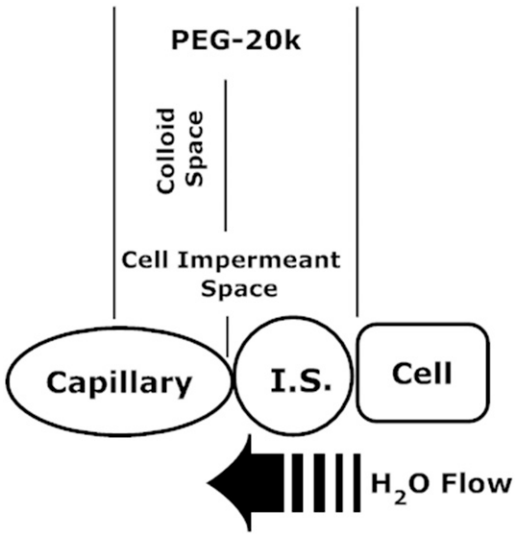

Fig. 1. Hypothetical mechanism of action for PEG-20k in LVR for shock. Cell impermeants can escape the capillary but are too large or charged to enter cells and thus create an osmotic gradient to prevent cell swelling during shock. We hypothesized that PEG-20k acts via biophysical effects on water movement through both cell impermeants and oncotic properties. To test our hypothesis, we tried to recapitulate the PEG-20k effect by combining a cell impermeant (gluconate) with a colloid (albumin). The movement of capillary water with oncotic agents increases capillary pressures that promote capillary flow even under low-volume states. The sum effect is to promote effective and efficient capillary transport and oxygen delivery in the low-volume state. I.S., interstitial space.

Materials and Methods

All animal work was conducted under a protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee, which is governed by the rules and regulations set forth in the National Institutes of Health guide and the United States Department of Agriculture.

Rodent Shock Model. An LVR model was used in adult rats to test the impermeant-based LVR solutions used for prehospital resuscitation during severe hemorrhagic shock (Parrish et al., 2015a,b). Adult male Sprague-Dawley rats were anesthetized and maintained in a light surgical plane of anesthesia with isoflurane during the study. Isoflurane was delivered through a nose cone with a fraction of inspired oxygen of 100%. The animals were allowed spontaneous respirations to control their own ventilation and carbon dioxide levels. Polyethylene catheters were placed in both femoral arteries for blood pressure monitoring and blood sampling, and a third catheter was placed in a femoral vein for fluid administration. Heparin (500 U/kg) was given i.v. to maintain catheter patency. A 1-cm midline incision was created to induce some soft tissue injury and for placement of an intra-abdominal temperature probe. Animals were kept at 38°C using a heating pad and an incandescent light source. Arterial blood pressure, heart rate, and temperature were continuously recorded using PowerLab (AD Instruments, Boston, MA).

After a 15-minute stabilization period, arterial blood was removed at 1 ml/min into a syringe to maintain a mean arterial pressure (MAP) of 30–35 mm Hg. More blood was withdrawn as the animal compensated, but a maximum hemorrhage limit of 60% of blood volume was set. Blood volume (ml) was estimated as weight (g) $\times$ 0.06 + 0.77 as previously described (Arora et al., 2012). A MAP of 30–35 mm Hg was maintained until the plasma lactate reached a value between 9 and 10 mM, as measured every 15 minutes with a handheld lactate analyzer (Lactate Plus, Nova Biomedical, Waltham, MA) and every hour with the ABL-800 blood gas analyzer (Radiometer, Copenhagen, Denmark). Once the target lactate was reached, an LVR equal to 10% of the estimated blood volume was given i.v. over 5 minutes using a syringe infusion pump. Thirty minutes after LVR, serial lactate measurements were taken until the lactate again climbed back to the 9- to 10-mM target because the low volume infusions temporarily lower or stall the accumulation of plasma lactate. The main outcome measured was LVR time, which was defined as the length of time from the start of LVR administration to the time when lactate climbed back to 9–10 mM. The LVR time is a surrogate for tolerance to the low-volume or shock state. It is the length of time that a patient can safely remain in the low-volume state until definitive care and resuscitation are needed, clinically, the "golden hour." At the end of the experiment, the animals were euthanized by Euthasol injection or by exsanguination under anesthesia. The maximum obtainable LVR time in this study was fixed to 240 minutes owing to an isofluorane exposure rule that limited exposure of the animals to that exposure duration. Figure 2 depicts the experimental protocol.

Fluids used for LVR were: 1) normal saline; 2) 15% gluconate in saline, a cell impermeant; 3) 10% albumin in saline; 4) 10% PEG-20k

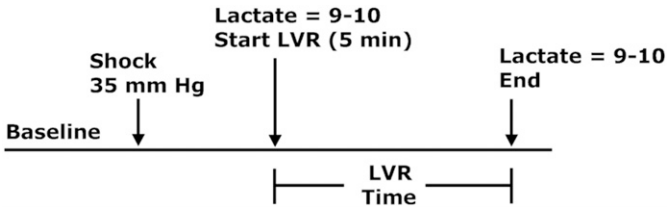

Fig. 2. Timeline diagram of the hemorrhagic shock and resuscitation protocol. Rats were hemorrhaged to a mean arterial pressure of 30–35 mm Hg. Once lactate reached 9 to 10 mM, LVR was administered. The primary outcome was LVR time, which is the length of time from start of LVR to the time at which lactate climbed back to 9 to 10 mM.

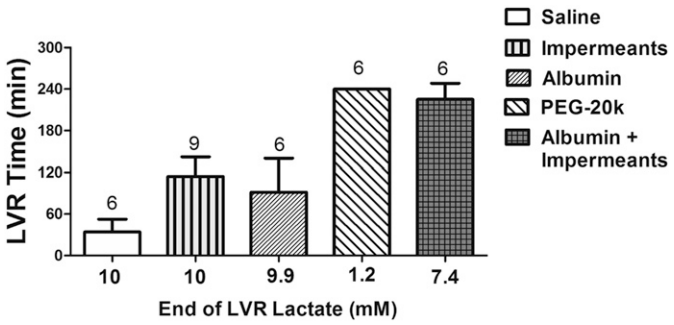

Fig. 3. The effect of different LVR solutions on LVR time. Data are presented as mean (S.D.). Numbers below bars indicate mean lactate at the end of LVR time, which by definition should be 9 to 10 mM. Numbers above bars indicate sample size. All treatment groups had significantly higher LVR times than the saline (control) group. No significant difference was seen between PEG-20k and the albumin + impermeant group.

in saline; and 5) 15% gluconate + 10% albumin in saline. Other outcomes recorded included the lactate at the end of the LVR time, which in most cases was 9 to 10 mM by definition. MAP was also recorded throughout the experiment. Test agents used for i.v. resuscitation (sodium gluconate, bovine albumin, and PEG-20k were obtained from Sigma-Aldrich, St. Louis, MO).

Regional Blood Flow. In another series of studies ($n = 6$), local capillary blood flow was studied using the colored microsphere technique (Adams et al., 2001; Parrish et al., 2015a). Animals were prepared as previously described, but a catheter was also placed into the aortic root using real-time pressure and pressure waveforms as indicators of catheter tip location by identifying the aortic valve. During the stabilization/baseline period, 300-μl colored microspheres (Triton Technologies, San Diego, CA) were rapidly injected into the aortic root as a calibrated arterial reference blood sample was simultaneously removed from the femoral artery catheter with a withdrawal pump at a constant rate of 0.25 ml/min. A different colored microsphere was injected 30 minutes after LVR. After the study, tissue samples were removed from the major organs, and microspheres were recovered from the tissue samples and reference arterial blood samples by alkaline digestion and repeated centrifugations. Dye coating purified colored microspheres was extracted with acidified 2-ethoxyethyl acetate and quantitated using a UV-visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Individual colors were resolved using a matrix inversion algorithm from the composite spectra. Blood flow was calculated by the tissue dye content using the reference blood draw as a blood flow standard. Correction for microsphere loss occurred using the recovery of blue microspheres that were added to the tissues as an internal standard before digestion (10,000 spheres added per sample). All flows were normalized to 100 g of tissue weight and expressed as the change from baseline values before shock and LVR.

Blood-Volume Determinations. Total blood volume of rats after shock and after various times after LVR was calculated using the indicator dilution technique (Iijima et al., 1998; Ertl et al., 2007). An i.v. bolus of fluorescein isothiocyanate (FITC)-albumin (Sigma-Aldrich, St. Louis, MO) of known volume and activity was administered and, a reference i.v. sample was taken 15 minutes later for estimation of the volume dilution effect. Plasma volume was calculated by the degree of FITC dilution using a standard dilution curve with saline. Plasma volume was divided by 1-hematocrit to determine the circulating blood volume. Blood volume was also assessed by the same indicator dilution principle using hematocrit during the LVR period. The assumptions of this method were: 1) that the red blood cells stay in the vascular compartment during LVR; 2) that the volume of the packed cell component remains constant during LVR (because no further bleeding is allowed); and; 3) that changes in hematocrit during LVR are inversely proportional to changes in the plasma volume component of the intravascular space. Baseline blood volumes before shock were estimated using a formula as previously described (Parrish et al., 2015a).

Circulating Cytokines. To determine a possible role for early type 1 T-helper cytokines in this model, we measured plasma levels of interleukin-1β (IL-1β) and tumor necrosis factor-α because they are representative of early type 1 T-helper cytokines that may be formed during hypovolemic shock and early resuscitation injury and play a role in later autolytic inflammation (Sato et al., 2008). Cytokines from plasma samples were determined by standard enzyme-linked immunosorbent assay using commercially available kits (Boster Bio, Pleasanton, CA).

Statistical Analysis. Data are expressed as mean ± S.D. Each group consisted of five to nine rats, which was derived from power analysis and the known variance of the data from similar studies. Data were analyzed by one-way or two-way analysis of variance and Bonferroni multiple comparison correction using the InStat program (GraphPad Software, Inc., La Jolla, CA). A P-value < 0.05 was considered statistically significant.

Results

The effects of a variety of chosen LVR solutions on LVR time are shown in Fig. 3. Normal saline was the control LVR fluid, which produced an LVR time of 34 ± 8 minutes. The LVR time significantly increased to 114 ± 10 minutes and 92 ± 20 minutes in the gluconate and albumin groups, respectively, compared with saline. The LVR time for the PEG-20k group was 240 ± 0 minutes ($P < 0.05$, relative to saline, gluconate, and albumin groups). This LVR time was cut off for technical reasons and would have been higher because the lactate at 240 minutes was only 1.2 mM, which is well below the target cutoff of 9 to 10 mM. No significant difference in LVR times was found between PEG-20k and the albumin + gluconate–treated groups (240 ± 0 minutes and 225 ± 24 minutes, respectively). The true comparison between these two groups is unknown, however, because the full LVR time in the PEG-20k group was not realized. This occurred because the lactate target was not reached owing to the duration of exposure of isofluorane anesthesia (i.e., 240 minutes). Technically, this result was attributable to time-dependent anesthesia problems in the animals after 4 hours. Therefore, the reported magnitude of the PEG-20k effect during shock in Fig. 3 is underestimated when measured by the LVR time.

Similar to the LVR times, the MAPs in the impermeant, PEG-20k, and albumin groups were much higher throughout the LVR period compared with the saline LVR control (Fig. 4). Generally, the MAPs during LVR correlated with the LVR times such that the groups with the longest LVR time (PEG-20k) also had the highest MAP and vice versa (Fig. 4).

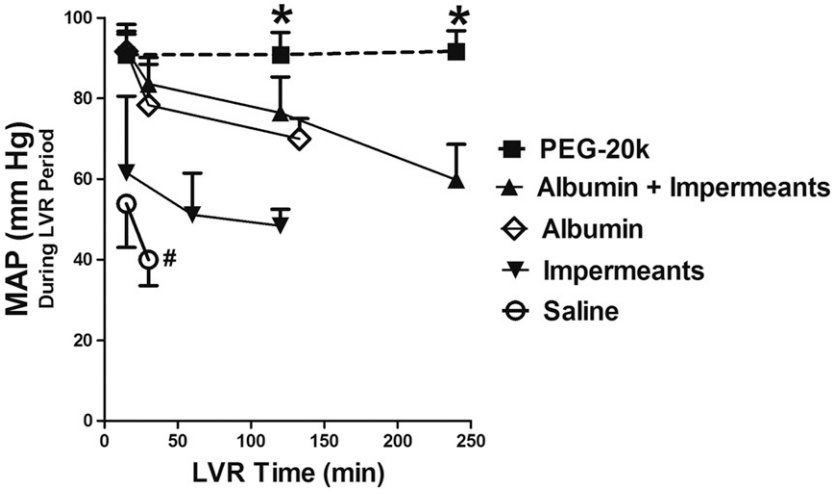

Fig. 4. MAPs after LVR administration, measured at 15 minutes, 30 minutes, throughout the LVR period, and at end of LVR time. $^{*}P < 0.05$ relative to the other values at the same corresponding times in the other groups. $^{\#}P < 0.05$ relative to all other values.

Reductions in capillary blood flow after shock and LVR, as measured by the colored microsphere technique, were significantly less in all organs and tissues (except the ileum) during the LVR period in PEG-20k resuscitated animals relative to the saline-resuscitated control animals (Fig. 5). All flow values (except in the left ventricle) in the saline group after LVR were statistically lower than their paired baseline values, and all flow values in the PEG-20k group after LVR were statistically unchanged from their paired baseline values. In other words, shock and LVR with saline caused significant reductions in local tissue blood flow, which was prevented when PEG-20k was used as the LVR solution.

Blood volume measurements made after hemorrhage and at 15, 30, and 60 minutes after LVR administration in the saline and PEG-20k groups are shown in Fig. 6. Blood volume was estimated before shock. Shock significantly reduced blood volume in both groups relative to baseline. Resuscitation with PEG-20k significantly increased blood volume at all times after LVR compared with the saline control LVR solution using either indicator dilution technique. Resuscitation with low volumes of 10% PEG-20k, but not saline, caused blood volume to increase significantly above values observed after hemorrhage.

Plasma levels of IL-1β and tumor necrosis factor-α at baseline, after hemorrhage, and 60 minutes after LVR are shown in Fig. 7. IL-1β concentrations after saline LVR were statistically higher compared with the corresponding levels after PEG-20k LVR. All other values were not significantly different either during the shock protocol for either cytokine or between treatment groups for any corresponding time points during the protocol for either cytokine.

Discussion

Our previous studies have demonstrated the efficacy of a novel platform of LVR crystalloid solutions in extending tolerance to the low-volume state or the amount of time that a severely shocked patient can safely remain in the low-volume state until definitive resuscitation and medical care are delivered. These solutions contain cell impermeants and are designed specifically to reduce the amount of ischemia-induced cell swelling during shock. LVR solutions that used the specific polymer PEG-20k produced striking biologic effects that increased the tolerance to the low-volume state

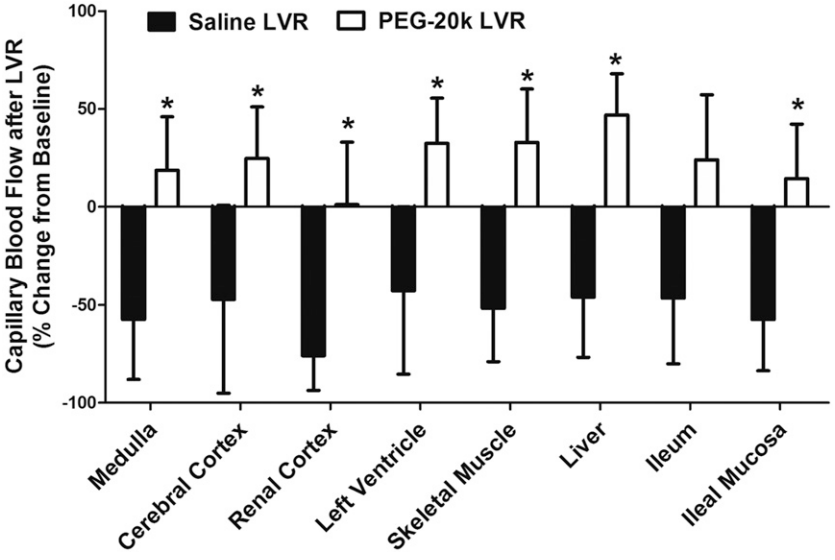

Fig. 5. Capillary blood flow measured 30 minutes after LVR. Data are presented as mean (S.D.) as percentage of change from baseline. Each rat served as its own baseline. $^{*}P < 0.05$ compared with the saline group. For the saline group, all flows except left ventricle were statistically less than paired baseline flows. For PEG-20k, all flows were not different from baseline. $n = 5$.

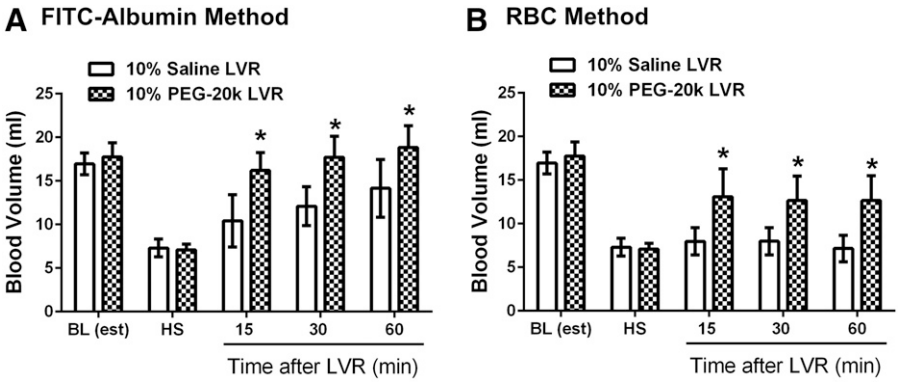

Fig. 6. Circulating blood volume measured in rats by the indicator dilution technique using a FITC-labeled albumin probe (A) or hematocrit (B) to estimate the size of the intravascular fluid compartment during LVR with saline or saline containing 10% PEG-20k. Blood volume values were estimated in the rats before shock (BL, baseline), and measured with indicator dilution after the hemorrhagic shock (HS) period, and after the LVR period. Values are mean ± S.D. for four rats in each group. $^*P < 0.05$ relative to the corresponding value in the saline group. Both LVR solutions were given at a volume of 10% of the estimated baseline blood volume. Saline was 0.9% NaCl solution, and PEG-20k was a 10% wt/vol solution of polyethylene glycol (mean mol. wt. = 20,000 Da) dissolved in saline.

(LVR time) 4- to 8-fold over previous classes of smaller cell impermeants or conventional saline-based resuscitation fluids. The objective of this study was to explore the mechanisms of action of PEG-20k that account for these strong biologic and preclinical effects.

Simple cell-impermeant molecules, like gluconate, trehalose, and raffinose, have been used in organ-preservation solutions to prevent tissues from swelling in cold ischemic environments. Ideal cell impermeants are molecules that have a unique size enabling them to freely escape the capillary space but not cross the cell membrane because they are too large or charged. Thus, they accumulate outside the cell and osmotically hold water from entering the cell, which is its normal propensity during shock and ischemia when energy-dependent volume-control mechanisms fail (Na^+/K^+ ATPase). These simple impermeants prevented cell and tissue swelling in rodent models of hemorrhagic shock when introduced into LVR solutions. Their low toxicity and chemical inertness allow them to be used successfully in high concentrations capable of exerting these necessary biophysical effects on water shifts during shock. Gluconate quadrupled the LVR time compared with saline. In an attempt to optimize this effect, we added colloidal molecules to create a second osmotic gradient in the microcirculation, which was designed to pull water into the capillary space. The first studies used PEG-20k as a colloid, together with the simple impermeant gluconate. This increased the LVR time 7-fold in the rodent model, which suggested that the double-gradient approach may have worked; however, subtraction experiments using only PEG-20k without the gluconate produced the same effect as the two together. It was then hypothesized that the larger PEG-20k molecule may be acting as both a cell impermeant and a colloidal molecule.

Further studies indeed determined that this was true since the osmotic reflection coefficient for PEG-20k was determined to be 0.5 in the rat microcirculation (thoracic and mesenteric beds) under nonshock conditions (Parrish et al., 2015b). This means that roughly a third of the PEG-20k escapes the capillary space to load into the interstitium, where it acts like a simple cell impermeant (gluconate) and two-thirds of the molecules in the circulation remains behind in the capillary space, where it acts as a colloidal agent to produce the second osmotic gradient. Although this double-gradient effect of PEG-20k is completely and unambiguously supported by the biophysical osmotic reflection coefficient data, the translation of these properties into the strong biologic effects seen with PEG-20k remains less certain. To make this biologic link and support the hypothesis that the second osmotic gradient indeed contributes to the very long LVR time of PEG-20k during shock, we attempted to recapitulate the biologic effect of PEG-20k with two distinct molecules used together: 1) a small ideal cell impermeant (gluconate) that produces an osmotic gradient between the intracellular and the extracellular space and 2) a classic colloidal agent (albumin) that produces a second osmotic gradient between the interstitial space and the capillary (intravascular) space.

Indeed, the use of these two distinct molecules (albumin and gluconate) produced a biologic effect during LVR that was similar to that when PEG-20k was used alone. This result supports the hypothesis that the biologic effect seen with PEG-20k may be due, in part, to its unique biophysical attributes that allow it to behave as both an ideal impermeant and a colloid in the microcirculation during shock states; however, since we do not know the exact LVR time with PEG-20k alone, because the period was terminated early, biologic differences between this group and the albumin + gluconate

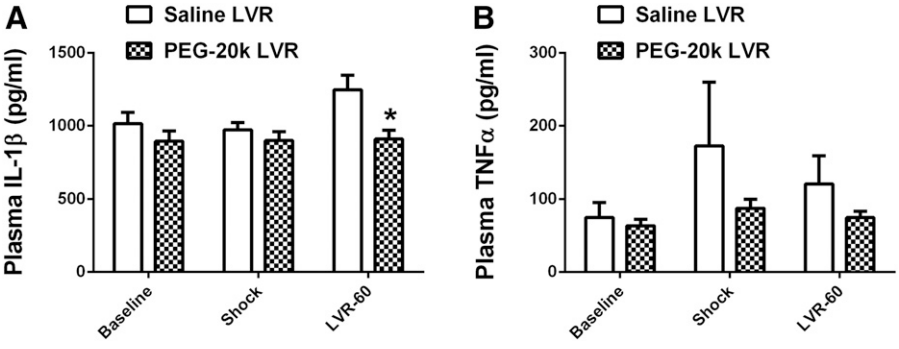

Fig. 7. Plasma IL-1β (A) and tumor necrosis factor-α (B) concentrations in rats at baseline after hemorrhagic shock and after 60 minutes of LVR (LVR-60). Rats received either a saline LVR or a PEG-20k LVR after shock equal to 10% of the calculated blood volume administered over 10 minutes. All values are mean ±S.D., $n = 6$. $^*P < 0.05$ relative to the corresponding saline value.

group probably do exist, which suggests that not all the PEG-20k effect may be attributable to osmotic gradients. Whereas other biologic effects of PEG-20k are likely in these settings, they currently remain unknown. Protection and hydration of the shock-eroded glycocalyx and induction of cell-surface immunocamouflage by PEG-20k polymers remain strong possibilities.

Rats receiving saline had a transient increase in MAP during LVR infusion, but the pressure started to drop as soon as the infusion stopped. This happens because saline physiologically distributes unequally between the vascular and interstitial compartments. About 20% of the administered saline volume will remain in the intravascular space, and 80% goes elsewhere (Haupt, 1986, 1989). Since only the 20% remaining in the capillaries supports arterial pressure, it is not surprising that saline given in low volumes during shock produce poor or absent effects on the arterial pressure. When PEG-20k–based LVR solutions are administered, it may prevent saline from loading into the interstitium while simultaneously returning the fluid that leaked into the interstitium back into the capillary spaces during the shock period (Gosling, 2003; Keel and Trentz, 2005; Kumar et al., 2010). These passive-volume shifts reduce local tissue swelling, decompress the microcirculation, reduce resistance to flow, and reload the capillaries. All these changes drive local tissue perfusion and provide cardiac preload as demonstrated directly by the increase in MAP and indirectly by the rapid clearance of lactate in LVR and by direct measurements of increased capillary blood flow with PEG-20k compared with saline. This is further supported by the significant effects of PEG-20k solutions on expansion of the plasma and blood volumes after their administration during the LVR period. PEG-20k maintenance of perfusion pressure and lactate clearance in the low-volume state can extend up to 8 hours (unpublished data), which was the longest period examined so far.

Hemorrhagic shock decreases oxygen delivery, which results in the accumulation of oxygen debt during the low-volume state. Using lactate clearance directly and the LVR time indirectly, which relies on lactate levels, we were able to demonstrate clearly that PEG-20k–based LVR solutions both stop accumulation of oxygen debt and rapidly repay the debt, even during the low-volume state. This is supported by the rapid drop in lactate levels after PEG-20k–based LVR and the extremely long LVR times relative to the values seen with conventional saline LVR. Whereas about 50% of this drop in lactate can be attributable to dilution from an expanding intravascular volume, much of the remaining lactate clearance may be due to increased efficiency of microvascular capillary oxygen transfer and/or an overall increase in oxygen delivery, which drives the conversion of lactate back to pyruvate for subsequent aerobic ATP synthesis. In fact, preliminary studies in a large animal porcine model of shock and LVR indicate that PEG-20k–based LVR under similar low-volume conditions leads to a hyperdynamic cardiovascular response characterized by cardiac output increasing 50% higher than preshock baseline values over much of the low-volume state (Plant et al., 2016). These combined factors likely account for the apparent rapid oxygen debt repayment and the 100% overnight survival (Parrish et al., 2015a) seen in PEG-20k–treated rodents. In patients with long prehospital transport times, this can limit further ischemic and reperfusion injury and possibly begin debt repayment during the transport period.

A likely mechanism of action of PEG polymers in LVR not addressed by this study includes reconstruction of the endothelial glycocalyx by PEG-20k. Shock and crystalloid resuscitation are known to erode the glycocalyx, thus promoting resuscitation injury by promoting cellular inflammation (Torres Filho et al., 2013; Torres et al., 2013). Polyethylene glycol polymers are known to bind to the cell membrane with their accompanying water layers (Neu et al., 2003) that could effectively rebuild the glycocalyx during the LVR and reperfusion period (Hauet and Eugene, 2008). Although this likely happens, it seems that such effects would be expressed after longer periods of resuscitation since cellular inflammation may require hours rather than minutes, which is the time period where rapid capillary blood flow and lactate clearance were observed with PEG-20k in this study. It is reasonable to suggest that PEG-20k LVR may reload peripheral capillaries by early osmotic water transfer while having later effects on glycocalyx-mediated cellular inflammation. Our cytokine data also support this.

In conclusion, 10% PEG-20k is a novel LVR fluid with encouraging potential for prehospital use in hemorrhagic shock. By improving local oxygen delivery and capillary perfusion, these solutions increase tolerance to the shock state during prolonged prehospital and transport periods. This study tests a likely biophysical mechanism for its efficacy, namely, the osmotic cell-to-capillary transfer of accumulated water that drives efficient local perfusion under low-volume conditions.

Authorship Contributions

Participated in the research design: Plant, Parrish, Limkemann, Mangino.

Conducted Experiments: Plant, Parrish, Limkemann, Mangino.

Performed Data Analysis: Plant, Mangino.

Wrote or contributed to the writing of the manuscript: Plant, Parrish, Limkemann, Ferrada, Aboutanos, Mangino.

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Chapter 8

CRYSTALLOID AND COLLOID RESUSCITATION: HYPERTONIC SALINE, STARCHES, POLYMERS AND GELATINS

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ABSTRACT

Traditional strategies to correct severe hemorrhagic hypotension relied on massive and aggressive fluid replacement, born from decades of animal experiments showing 2-3-fold crystalloid replacement was necessary in irreversible shock. However, crystalloid overloading can cause serious compromise in metabolism, inflammation, coagulation, and can also further exacerbate bleeding. This approach has more recently been replaced by low volume and permissive hypotensive resuscitation, which produces superior outcomes. Clinical studies further support the avoidance of crystalloids altogether early during hemorrhagic shock in favor of blood

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product administration in 1:1:1 ratios of FFP:packed red cells: platelets. Yet, since blood products are not practical in pre-hospital or austere settings, a new approach and mechanistic understanding of stable low volume crystalloid resuscitation is needed. The use of low volume crystalloids containing hypertonic saline, hydroxyethyl starch, or gelatin polymer molecules designed to oncotically expand the plasma volume are theoretically appealing but have yielded disappointing clinical results. A new paradigm has emerged describing a key mechanism of resuscitation injury involving cell and tissue swelling secondary to ischemia-induced loss of cell volume control. Novel low volume resuscitation solutions directed at this perturbed mechanism that contain cell impermeant polymers of polyethylene glycol are orders of magnitude more effective than conventional solutions at extending the golden hour after high-volume hemorrhage in pre-clinical models. These next generation crystalloids may hold promise for resuscitation in the military and civilian pre-hospital settings and in the emergency departments and operating rooms where prolonged tolerance to the low volume state is needed.

INTRODUCTION

The first written record of intravenous salt solution infusion was by Dr. Robert Lewins in 1832, during a cholera outbreak in England. Dr. Lewins gave credit to Dr. Thomas Latta for the actual intravenous injection, a treatment on advice from a physician scientist named Dr. William Brooke O'Shaughnessy [1]. Dr. O'Shaughnessy studied the blood of cholera patients and found "(the blood) has lost a large proportion of its water…it has lost also a great proportion of its neutral saline ingredients" [2]. He advised that blood needed to be restored to its natural specific gravity and of its "deficient saline matters." O'Shaughnessy further advised that these could be effected by oral fluid intake or by intravenous injection of "tepid water holding a solution of the normal salts of the blood" [1]. After Dr. Lewins' and Dr. Latta's description of the practice, several reports of treatment with intravenous saline followed. According to Cosnett, "almost all reported dramatic, but often temporary, improvement, but relapse was frequent as the purging continued." Modifications of IV fluids were also attempted, including administering boiled, skimmed, and strained milk [1, 3]. Intravenous saline therapy has come a long way, but even now, modifications of IV treatments continue to be studied.

In the 1960s, Shires published studies on the resuscitation of hemorrhagic shock, concluding that crystalloids, along with blood, were important in survival after hemorrhagic shock [4]. After restoring shed blood or shed blood plus extra

plasma in a controlled hemorrhage model, Shires found that extracellular fluid was salt-deficient as compared to that pre-shock. He suggested that the deficiency of interstitial fluid was a result of fluid redistribution during shock. His studies revealed higher survival rates in the animals receiving shed blood plus crystalloid volume, which, he theorized, replaced the interstitial fluid deficit. We now understand that redistributed interstitial fluid is entering cells during shock [5, 6] causing cell swelling. However, after Shires' study, large volume crystalloid resuscitation became the norm and was practiced widely in the Vietnam Conflict. A high incidence of the acute respiratory distress syndrome (ARDS) was recognized in Vietnam War soldiers [7] after the Denver group first reported the phenomenon in civilians [8] and described its clinical state, pathological features, and exacerbating factors including large volume crystalloid resuscitation. Despite these reports, this practice persisted until decades later when early, aggressive resuscitation was questioned as unsafe in actively bleeding trauma patients [9]. Since then, many other studies have found aggressive fluid resuscitation to contribute to the lethal triad of hypothermia, acidosis, and coagulopathy, as well as increased bleeding, abdominal compartment syndrome, and overall increased mortality [10-12].

Not only the volume, but also the type of resuscitation fluid has changed throughout the decades [13]. In this chapter we review different types of new and old resuscitation fluids, discuss the evolution of low volume resuscitation, and introduce novel fluids currently being actively investigated.

Low Volume Resuscitation: Up until the 1960s, aggressive crystalloid resuscitation in the pre-hospital or emergency room setting was not advocated for fear of "popping the clot" prior to definitive hemostasis, increasing both loss of intravascular volume and need for further resuscitation [14]. Concerns had been raised during World War I, when Cannon stated "Injection of a fluid that will increase blood pressure has dangers in itself. If the pressure is raised before the surgeon is ready to check any bleeding that may take place, blood that is sorely needed may be lost" [15]. While these sentiments were reiterated in World War II, it would be decades before "damage control" surgery (DCS) strategies would become widely accepted as a result of military experiences in Iraq and Afghanistan [16]. Goals of DCS are to [1] obtain hemostasis, [2] minimize iatrogenic resuscitation injury, and [3] prevent worsening shock and "the bloody vicious cycle" of traumatic coagulopathy, profound acidosis, and hypothermia [16]. More recently damage control resuscitation (DCR) has been proposed to accompany DCS principles in acutely hemorrhaging patients [See Chapter 7].

Other potential adverse outcomes associated with over-resuscitating critically ill patients with crystalloids emerged four years after Shire's landmark study [4], which included hypernatremia, hemodilution, and (most importantly) "intracellular swelling in response to the low flow state" [17]. The authors implored physicians to be judicious in the use of crystalloid resuscitation, writing, "*The objective of care is restoration to normal physiology and (…) function of organs with a normal blood volume, functional body water and electrolytes. This can never be accomplished with inundation.*"

However, these pleas were ignored despite growing evidence through the 1970s and 80s that excessive crystalloid resuscitation could be quite deleterious. In a 2006 review of the cellular, metabolic, and systemic consequences of aggressive resuscitation strategies [12], the following concerns were identified:

1) ***Cellular and inflammatory disturbances***: Cellular swelling due to both shock and exogenous crystalloid may overwhelm regulatory mechanisms and cause a cytosolic acidification, inactivation of important enzymes and signaling pathways, and release of inflammatory mediators [18-20] thought to contribute to post-resuscitative injury including ARDS, multi-organ failure, and abdominal compartment syndrome. Secondary abdominal compartment syndrome due to liberal crystalloid use is associated with mortality rates consistently exceeding 50% [21].
2) ***Pulmonary complications***: pulmonary edema is related to both a decrease in colloid oncotic pressure (i.e., a 50% drop in oncotic pressure increases endothelial fluid flow by four-fold) and aggressive crystalloid administration both linked to increased morbidity and mortality in critically ill patients [22, 23].
3) ***Cardiac complications***: Based on Starling principles of myocardial performance, excessive resuscitation past the optimum point of stroke volume and contractility responsiveness can paradoxically decrease cardiac performance similar to a state of congestive heart failure, seen with cardiac or renal insufficiency. Outcomes include impairment of pulmonary gas exchange and tissue oxygenation as well as increased rates of arrhythmias with greater mortality in patients receiving early saline-based regimens of resuscitation instead of blood-products [24]. In a 2003 multicenter RCT, reduced perioperative IV fluid regimens decreased cardiopulmonary complications after elective colorectal resection [25].

4) ***Gastrointestinal [GI] disturbances***: critically ill patients with GI dysfunction have increased risk of ventilator-associated pneumonia, longer ICU and hospital stays, and lower survival rates. This is compounded by excess fluid administration resulting in splanchnic edema, worsened respiratory function, increased bacterial translocation, impaired wound healing, and higher rates of sepsis, multisystem organ failure, and death [26-28].
5) ***Coagulation disturbances and exacerbation of hemorrhage***: rapid and excessive fluid administration contributes to a dilutional coagulopathy [29], increased hemorrhage volume, decreased oxygen delivery, and increased mortality [30, 31].

Resuscitation strategies that *slow, delay, or limit fluid administration* in the pre-hospital and perioperative settings have been shown to drastically reduce these adverse outcomes and curb mortality in animal models [32, 33]. However, less consensus exists among human trials with regards to timing, volume, and rate of resuscitation strategies due to variability in study designs, risk of selection bias, and clinical heterogeneity [34].

A prospective 1998 cohort study [35] of patients admitted to a level I center with traumatic hypotension (systolic pressure less than 90 mmHg) showed a clear association between increasing volumes of crystalloid use in the first 24 hours after injury and rates of mortality, infection, and organ failure. While it was clear that pre-hospital hypotension contributed to a 54% mortality rate, the direct causal link between mortality and fluid resuscitation was not proven, only correlated.

The detrimental effects of aggressive pre-hospital resuscitation have since been demonstrated in several retrospective cohort or matched analyses [36], confirming a greater preponderance of abdominal compartment syndrome [37], coagulopathy [38], and mortality [39, 40]. In a 2013 retrospective matched pairs analysis (n = 1896) [40] of multiply injured trauma patients with ISS ≥16 and SBP ≥60 mmHg, higher survival rates were reported in patients with low volume (0-1500 ml) versus traditional or high volume pre-hospital resuscitation [>1500 ml].

A landmark randomized human trial was published by Bickell et al. in 1994 that showed survival benefits from limited or delayed resuscitation (prior to definite surgical hemostasis) in penetrating torso injury associated with shock [9]. Mortality was 62% for the delayed resuscitation group receiving an average volume of 375 ml prior to surgery, compared to a 70% mortality (p = 0.04) in those resuscitated immediately in the prehospital setting and

receiving an average 2,478 ml pre-operatively. As this study was limited to young patients with penetrating injuries and evacuation times under 30 minutes, the effectiveness of permissive hypotension or delayed resuscitation could not be extrapolated to other populations of hemorrhaging patients experiencing ongoing insults from shock and oxygen debt without lifesaving intravascular rescue [41, 42].

The first propensity-adjusted prospective cohort analysis of goal-directed pre-hospital resuscitation in blunt trauma [43] evaluated patients with ISS > 15 (n = 1,216) and divided them into "HIGH" (>500 cc) or "LOW" (≤500 cc) prehospital crystalloid administration. They then further categorized them into groups with or without hypotension (SBP <90 mmHg). Interestingly, high volume prehospital crystalloid was associated with a two-fold increased risk of both 30-day mortality and acute coagulopathy, but only in patients without hypotension. The authors suggested that prehospital crystalloid resuscitation be goal-directed based on the presence of hypotension, echoing another similar study's recommendations [39].

A 2011 study [44] assessing goal-directed intraoperative resuscitation regimens randomized penetrating injury patients to groups with targeted minimum mean arterial pressures (MAPs) of either 50 mmHg or 65 mmHg. They found no survival differences between groups, although final average MAPs did not differ between groups (64.4 vs 68.5, p = 0.15). The higher MAP targeted group received more IV fluids and blood transfusions overall, which was associated with higher 24h mortality and coagulopathy rates. Similarly, in a 2002 study [45] where hemorrhagic shock patients were randomized to conventional (SBP > 100 mmHg) or permissive (>70 mmHg) blood pressure targets, no survival benefits were found in either group though study methodology was imperfect [13]. These studies demonstrated that systolic blood pressure end-points are difficult to achieve due to the dynamic interaction among fluid administration, anesthesia, and that the patient's autoregulatory mechanisms in shock, and hypotensive resuscitation is unlikely to have an impact on the in-hospital mortality of trauma victims in modern day practice [46].

A 2013 retrospective multi-institutional analysis [47] was undertaken to assess the impact of 24-hour crystalloid volume (ED + OR + ICU) on mortality and other outcomes in perioperative trauma patients managed with damage control laparotomy and massive transfusion protocol (10+ units pRBCs) with high ratio resuscitation (1:1-2 FFP:pRBCs), hypothesizing crystalloid overload would dilute beneficial effects of blood component replacement therapy. Results were consistent with previous studies showing increased survival in

patients receiving high ratio (e.g., 1:1) vs low ratio (e.g., 1:3) blood product resuscitation, with potential protective effects of FFP. However, it was the first multi-institutional study to show a fourfold increased morbidity from 24-hour crystalloid use in excess of 5-10L in the clinically preferred high ratio resuscitation (1:1) group related to deleterious cellular and molecular effects after trauma and hemorrhagic shock. Thus, these authors strongly cautioned overzealous and unnecessary use of crystalloid therapy (>5L/24 hours) in patients with severe hemorrhage undergoing MTP. However, blood products may not be available in the field or pre-hospital setting, where crystalloid therapy is often used.

A 2015 pilot RCT study by Schreiber et al [48] was designed to test "battlefield strategy in the civilian setting," and assess the feasibility and safety of "controlled resuscitation," (CR) in hypotensive trauma patients SBP ≤ 90 mmHg. Randomization was in the pre-hospital setting, and resuscitation was continued until hemorrhage control or 2 hours into hospitalization. CR was defined as giving 250 ml bolus if no radial pulse or if a systolic BP < 70 mmHg was present, with additional 250 ml boluses administered to maintain a radial pulse or SBP ≥ 70 mmHg. The "controlled" strategy resulted in a mean of 1.0 L (±1.5) administered volume, which was about half the volume administered with standard practices (2.0 L [±1.4]). Despite this, no differences were found with respect to ICU-free days, ventilator-free days, renal injury, or renal failure. However, 24h mortality was three-fold greater in the standard volume group than the controlled strategy group. The mortality difference was solely found in the blunt trauma cohort, not the penetrating injury group. The authors commented that these were preliminary pilot results not powered to detect important survival differences between groups. Despite methodological concerns, this study underscored the fact that large volume crystalloid is not advantageous, and that a period of hypotension may be reasonably well tolerated.

Two meta-analyses reviewing the timing and volume of fluid administration for bleeding patients failed to demonstrate an advantage of delayed vs early resuscitation, or of smaller vs larger volumes of fluid administration [49]. However, a 2014 analysis [34] of four RCTs (n = 798) and seven retrospective observational studies (n = 13,687) presented data suggestive of a mortality benefit from using restricted volume replacement strategies in pre-hospital penetrating trauma patients without TBI.

Thus current data available suggests that excessive resuscitation in hemorrhagic shock results in multiple deleterious effects. As such, "low volume resuscitation" is now recommended (Grade 1B) by both civilian and military

trauma groups as a part of a DCR strategy to maintain adequate perfusion without exacerbating hemorrhage [36]. This concept may be broken down into two slightly different strategies to prevent clot disruption and dilutional coagulopathy: *delayed resuscitation*, where fluids are withheld until bleeding is definitively controlled, and *permissive hypotension*, where fluid is given, but the resuscitative endpoint is less than normotension [13, 50]. For trauma patients with major bleeding but without brain injury, systolic blood pressure goal is 80-90 mmHg (Grade 1C) [36]. For trauma patients with major bleeding and severe traumatic brain injury (GCS ≤ 8), a MAP ≥ 80 mmHg is recommended (Grade 1C) to ensure adequate brain perfusion, as even a single episode of hypotension in these patients may cause a doubling in mortality [51]. Other factors such as blunt vs penetrating trauma mechanisms and proximity to a hospital need to be considered, but in the setting of ongoing hypotensive hemorrhage in the pre-hospital setting without access to blood products, goal-directed limited resuscitation with low volumes of isotonic crystalloids is advised to decrease both short- and long-term morbidity and mortality related to ischemic cellular edema and widespread fluid overload contributing to acidosis, dilutional coagulopathy, sepsis, and organ failure.

Types of Crystalloids and Colloids

For decades, physicians have based their selection of resuscitation fluids on the classic compartment model of fluid exchange. Extracellular water and electrolyte loss (due to third spacing or insensible losses) may be replenished with crystalloids, while whole blood loss or massive fluid shifts from vasodilation (e.g., burn) may be restored with oncotic colloids in lower volumes due to their enhanced ability to remain in the intravascular space [52]. Recent descriptions, however, have expanded Starling's model with the introduction of the glycocalyx concept of transvascular fluid exchange [53]. The glycocalyx is a web of membrane-bound glycoproteins and proteoglycans on the luminal side of endothelial cells that serves to maintain vascular integrity by producing a protective barrier that is now considered to be an integral determinant of transcapillary flow and membrane permeability [54]. When damaged, it functionally contributes to vascular "leakiness," as seen in shock or severe inflammation [55], surgery or trauma [54, 56]. Thus, restoration of glycocalyx integrity must now be considered when managing resuscitation of hemorrhagic shock [57, 58]. This has recently been documented with plasma containing resuscitation solutions [59]. Table 1 lists the compositions of common colloid

and crystalloid fluids, whereas Table 2 summarizes their major attributes including indications, pros, and cons.

Physiological Salt Solutions: The term "crystalloids" describes "aqueous fluids that contain crystal-forming elements [electrolytes], which easily pass through vascular endothelial membrane barriers followed by water" and equilibrate between compartments [60]. However, after less than one hour, about 25% of infused isotonic crystalloid volume remains in the intravascular space [52], directly contributing to interstitial and cellular edema. This fraction is actually higher for low infusion rates and decreases with the infusion time [61]. Hemorrhage reduces crystalloid elimination by 25-50%, even when hypovolemia is quickly corrected [61]. Crystalloid half-life is longer than that of colloids (6% dextran and 5% albumin), resulting in an increased risk for fluid overload in hemorrhagic shock [62]. Indeed, urine output (UOP) monitoring for signs of overload may be useless, with third-spacing into nonfunctional spaces unavailable for renal clearance (e.g., injured tissue or intracavitary compartments). While clearance rates may return to normal within about four hours of elective surgery, trauma or emergent surgery will prolong reduced clearance rates for days. Thus, tight control and restraint of crystalloid administration is paramount from the beginning of hemorrhage as to avoid iatrogenic morbidity.

"Normal" saline (NS, 0.9% NaCl) is a misnomer first coined in 1882 by Hartog Hamburger, referring to an incorrectly estimated weight/volume concentration of 0.9% instead of the physiologically normal 0.6% [54, 60]. Due to equal hypertonic amounts of sodium and chloride relative to normal plasma levels (Table 1), large NS infusions contribute to hypernatremia, dilutional hyperchloremic acidosis [63, 64], and metabolically-induced renal vasoconstriction [65]. *NS may thus be contraindicated in critically ill patients with compromised renal function or existing acidosis, such as those with shock.*

Alternatives to NS include "balanced" or "physiologic" crystalloids [64], which more closely resemble the electrolyte concentration of blood plasma (Table 1). However, none of the proprietary solutions are either truly balanced or physiologic. While the most popular balanced solutions are lactated Ringer's (LR), Hartmann's solution (HS), and Plasma-Lyte (PL), newer solutions continue to emerge, including colloid-based balanced vs. saline formulations.

Table 1. Summarizes the composition of the most common crystalloids and colloids [60]

Solute [mEq/L]	**Plasma**	**COLLOIDS**				**CRYSTALLOIDS**			
		4% albumin	6% HES 130/0.4 [Hetastarch]	Dextran-40 [10% solution]	Gelatin [Gelo-fusine]	Normal saline [NS]	Ringer's lactate [LR]	Hartmann's solution [HS]	Plasma-Lyte [PL]
pH	7.35-7.45	6.7-7.3	5.5	3.5-7.0	7.4	5.0	6.5	5.0-7.0	7.4
Na^+	135 - 145	148	154	154	154	154	130	131	140
K^+	4.0 - 5.0	0	0	0	0	0	4.5	5	5
Ca^{2+}	2.2 - 2.6	0	0	0	0	0	2.7	4	0
Mg^{2+}	1.0 - 2.0	0	0	0	0	0	0	0	1.5
Cl^-	95 - 110	128	154	154	120	154	109	111	98
Acetate	0	0	0	0	0	0	0	0	27
Lactate	0.8 - 1.8	0	0	0	0	0	28	29	0
Gluconate	0	0	0	0	0	0	0	0	23
Bicarbonate	23 - 26	0	0	0	0	0	0	0	0
Osmolarity [mOsm/L]	291	250	286 - 308	308	274	308	280	279	294
Colloid [g/L]	35 - 45	20	60	100	40	0	0	0	0

Table 2. Summarizes major attributes of common colloid and crystalloid solutions

Fluid	Indications	Pros	Cons
CRYSTALLOIDS	Extracellular fluid loss	-Inexpensive -No coagulopathy -No allergic reaction	-Limited intravascular expansion [<25% volume] -Tissue edema/overload
Normal Saline [NS]	Hyponatremia, hypochloremia, metabolic alkalosis	-Cost -Availability -Provider familiarity	-Hyperchloremic acidosis -Hyperkalemia [cell shifts responding to acidosis] -Nephrotoxicity
Physiologic solutions: LR, HS, PL	Critically ill: -Surgery -Trauma -DKA Primary fluid for U.S. civilian use in absence of blood	-Physiologic balance -No hyperchloremic acidosis or AKI compared to saline	-Hypotonic [avoid in TBI] -LR: hyperlactatemia [sodium lactate, not lactic acid] in liver failure
Hypertonic Saline [HTS] ± Dextran [HTD]	No clear indications since the trials were stopped for ineffectiveness in hemorrhage and TBI	-Rapid ↑BP. -Anti-inflammatory	-Hypernatremia -Coagulopathy [dextran] - Ineffective
COLLOIDS	Intravascular volume expansion	-Rapid ↑BP.	-No overall proven benefit over crystalloids
Albumin	Severe burns, paracentesis	-Generally safe -Benefit in septic or distributive shock	-Cost -↑Mortality in TBI
Hetastarches [HES]	Primary fluid for military use in absence of blood	-Anticoagulant effect	-Renal impairment/RRT -Accumulation in liver/skin -Coagulopathy
Dextrans	Combination with hypertonic saline	-Anticoagulant effect	-Impaired cross matching -Coagulopathy
Gelatins [Not in US]	Hypovolemia	-Anticoagulant effect	-Anaphylaxis -Coagulopathy

Several head to head comparisons of normal saline [chloride-liberal] to "balanced" (chloride-restricted) solutions have been performed. In a 2012 observational study [66] of patients undergoing major open abdominal surgery, balanced crystalloids resulted in lower in-hospital mortality (2.9% vs 5.9%, $p < 0.0001$) and fewer major complications (23% vs 33.7%, $p < 0.0001$), including post-operative infections, renal failure requiring dialysis, blood transfusions, acidosis, and interventions. In a 2014 prospective matched cohort study [67] comparing chloride-rich (e.g., normal saline, Gelofusine, 4% albumin in NaCl) to chloride-restrictive solutions (e.g., lactated Hartmann's, balanced Plasma-Lyte, or chloride-poor 20% albumin) in critically ill patients, they found significantly lower AKI rates and use of renal replacement therapy in those receiving chloride-restrictive fluids. A small RCT in trauma hemorrhage [68] showed no mortality benefit of PL to NS resuscitation for the first 24 hours after injury in patients requiring blood transfusion, intubation, or operation within 60 minutes of hospital arrival. While PL did show a mean improvement in base excess over NS, there were also no differences between groups with regard to administrated volumes, UOP, or resource utilization. A secondary analysis of this study [69], demonstrated a net cost benefit in using balanced solutions.

The replacement of lower concentrations of the chloride, as with lactate in LR was first proposed by Dr. Hartmann in 1932 to curb effects of acidosis through hepatic conversion to bicarbonate [13, 70]. There is little evidence that the lactate buffer within LR causes any physiologic derangement and may serve as a metabolic fuel substrate [71]. However, as LR is still a hypotonic fluid, it should be avoided in patients with TBI in order to minimize fluid shifts into damaged cerebral tissue [36]. Additionally, other solutions that restore pH (like Ringer's acetate) may be advantageous in rapidly ameliorating splanchnic dysoxia [72], and potentially the development of sepsis and multi-organ failure, and death [26].

In conclusion, as balanced salt solutions have shown few harmful effects, NS and other chloride-rich solutions should be restricted in the critically ill bleeding patient as they may cause hyperchloremic metabolic acidosis, renal vasoconstriction, acute kidney injury, and in rare cases need for renal replacement therapy. Balanced salt solutions are increasingly recommended as first line crystalloids for patients with hemorrhage, undergoing surgery, or with diabetic ketoacidosis [54]. The 2016 European Guidelines on the management of major traumatic bleeding [36] recommend isotonic crystalloid solutions be initiated in the hypotensive bleeding trauma patient (Grade 1A) in a restricted fashion with targeted blood pressures (Grade 1C), as described above. They also recommend avoidance of excessive use of 0.9% saline in any patient (Grade 2C)

and hypotonic solutions like LR in patients with severe head trauma (Grade 1C). It remains unclear, however, which specific isotonic balanced crystalloids are preferred in this patient population [71].

***Hypertonic Saline (with and without Dextrans)*:** Hypertonic saline may intuitively seem like an ideal low volume resuscitation fluid as it causes fluid shifts that increase intravascular volume, increases cardiac output and thus oxygen delivery. In early animal studies, hypertonic saline (2400 mosmol/l) given to canines in severe hemorrhagic shock increased blood pressure, cardiac output, and mesenteric flow resulting in 100% short term and long term survival as compared to 0% of counterparts receiving normal saline [73, 74]. Hypertonic saline was also found to have beneficial immunomodulating effects by enhancing or restoring immune function after hemorrhagic shock [75] and limiting neutrophil activation and sequestration as well as upregulating anti-inflammatory cytokines [76, 77].

Dextrans are plant-derived polysaccharide colloids with molecular weights greater than 1000 Dalton that increase intravascular oncotic pressure (see below). Early studies of dextran resuscitation showed improvements in blood flow and oxygen consumption by increasing vascular volume and reversing "microcirculatory sludge" through hemodilution [78]. The addition of dextran to hypertonic saline was to theoretically prolong the osmotic effect of hypertonic saline. Unfortunately, clinical trials with hypertonic saline [HS] or hypertonic saline plus dextran (HSD) confirmed that these fluids were safe, but with the exception of one study, there was no statistical improvement in survival [79]. In 1991, Mattox et al. randomized hypotensive trauma patients to either hypertonic saline with dextran or isotonic crystalloid. They found that the HSD group had significantly higher blood pressure, and that patients in the HSD group requiring surgery had improved survival, but that there was no difference in overall survival. The authors concluded that HSD was safe, but not superior [80]. In a subsequent randomized, double-blinded study of blunt trauma patients with shock, HSD also did not improve survival compared to patients receiving lactated Ringer solution [81]. Interestingly, the HSD group received a significantly higher volume of prehospital fluids as providers were given discretion to give additional LR fluid as needed during transport. The study was closed early due to futility [81]. A meta-analysis of several studies suggested an improvement in survival to hospital discharge [82].

The Resuscitation Outcomes Consortium (ROC) trials have been the largest randomized clinical trial of HS and HSD after hemorrhagic shock [83] and TBI [84]. Both studies were underpowered and unable to show a difference in mortality and were stopped early due to futility and safety concerns [83].

Another possible reason these studies did not show superiority was that the clinical dose was a set volume, rather than a weight-based dose, as described in most of the successful animal studies [79]. So whereas the usual animal study dose was 4 ml/kg, the 250 ml dose in the clinical trials may have been ineffective depending on the patient's weight. Some investigators suggested that HS or HSD rendered to patients an appearance of adequate perfusion due to improved blood pressure, which may have prevented early providers from recognizing or treating hemorrhage. The end result of hypertonic saline administration at the cellular level is that cells will bear the burden of the extra sodium load, through increased intracellular water and cell swelling. Cell swelling, in turn, compresses capillaries and hinders microcirculatory flow [85, 86]. This chain of events may be a cell-based explanation of why hypertonic saline has not proven to be effective in clinical trials.

Interestingly, a subset of patients from the hemorrhagic shock ROC trial had blood drawn for coagulation and fibrinolytic biomarkers. This subsequent study found that hypertonic solutions, especially HSD, resulted in hypocoagulability and hyperfibrinolysis [87]. Thus, due to its coagulopathic risks and for the cell physiology reasons listed previously, hypertonic saline solutions are unlikely to be effective low volume resuscitation fluids.

Hydroxyethyl Starch: Hydroxyethyl starches (HES) are plant-derived glucose polymers (i.e., large carbohydrate molecules) that have been chemically modified to resist degradation. Since its introduction in the 1970s, due to the limited availability and relative expense of albumin, several HES products have been developed, differing in physicochemical properties such as molecular weight (e.g., 130, 200, 450 kDa), degree and pattern of hydroxyethylation (i.e., molar substitution, e.g., 0.4, 0.5, 0.7), and concentration (e.g., 6%, 10%) [54]. HES solutions have been used widely in patients undergoing major surgery, in critically ill ICU patients, and primarily as the first-line resuscitation fluid in the military though this practice has greatly decreased in recent years.

HES is either excreted in the urine or taken up by tissues, with estimates that 26-42% may reside in whole-body tissue 24h after infusion [88, 89]. Accumulation of HES within the reticuloendothelial has been directly implicated in adverse events and toxicity occurring in the skin, liver, and kidney. This effect relates to HES of higher concentrations (10%), molecular weights (200 kDa), and molar substitutions (0.5). However, even newer 6% HES (130/0.42) has been associated with increased rates of acute kidney injury, renal replacement therapy (21-35%), and mortality in severe sepsis and critically ill ICU patients [60, 90]. The Crystalloid versus Hydroxyethyl Starch Trial (CHEST) study [91] evaluated 7000 patients, and in subgroup analyses, found

no differences in 90-day mortality between HES and saline for patients with sepsis, trauma, or TBI. Interestingly, lower rates of new cardiovascular failure (RR 0.91, CI 0.84-0.99) were found with HES, which was also associated with higher rates of hepatic failure (RR 1.56, CI 1.03-2.36). Thus, these and several other studies strongly suggest that HES administration results in increased rates of renal failure requiring replacement therapy, and new-onset liver injury.

Additionally, the use of HES has been associated with alterations in coagulation, specifically, changes in viscoelastic measures and fibrinolysis resulting in a weaker and smaller clot [92]. Decreased levels of VIII and von Willebrand Factor (VWF) have been reported in healthy volunteers and surgery patients, and HES may not only affect clot formation through alterations in the thrombin-VIII-fibrin axis, but also platelet function through altered expression and activation of GPIIb-IIIa [93]. Reductions in concentrations of other plasma coagulation factors have also been reported and ascribed to plasma dilution and altering platelet function through physiologic shrinkage, but there is also likely a pharmacologic effect again correlated with the size and weight of starch due to elimination kinetics.

Despite these results, clinical consequences of the hypocoagulable effects in specific patient populations such as in surgery or trauma remain unclear. The high molecular weight Hetastarch – the only HES solution approved for use as plasma expander in the United States – was associated with increased post-operative bleeding after neurosurgery [94] and cardiothoracic surgery [95] in studies from the 1980s-1990s. While several in vitro studies have shown effects on increasing fibrinolysis, one study in cardiac surgery patients using much smaller HES did not [96]. A 2011 systematic review of HES 130/0.4 on hemostasis [92] found that 65% of measured hemostasis variables demonstrated significant hypocoagulation as compared to control groups. They concluded that until results from more well-designed RCTs were available, “safer fluids should be chosen for patients with coagulopathy.”

A large body of HES literature from one author has now been found to be of “suspected lack of integrity” and as such consensus and metaanalysis studies have sought to remove these studies from their analyses. Three different 2014 meta-analyses excluding retracted studies have since been performed and while two found increased kidney injury and mortality in critically ill patients with sepsis treated with HES [97], another showed no differences [98].

In trauma, one small (n = 115) RCT [99] called the FIRST trial (Fluids in Resuscitation of Severe Trauma) showed a benefit of HES in penetrating trauma (n = 67), with HES (130/0.4) providing significantly better lactate clearance and less renal injury than saline up to the first post-resuscitation day. HES (5.1 ± 2.7

liters) and NS (7.4 ± 4.3 liters) showed similar results in the blunt trauma group, but the HES group was significantly more injured and required more blood products.

In conclusion, while some controversy still remains (especially in elective perioperative patients), HES use in the critically ill has definite associations renal, hepatic and coagulation dysfunctions and are no longer recommended as a first line resuscitation fluid [60, 90].

Albumin: Colloids were first introduced during World War II after the development of blood fractionation in 1941, when purification of human albumin was given in large quantities for resuscitation of severe burn and trauma patients at Pearl Harbor [54, 100]. Since then, several concentrations of albumin have become available ranging from 4-25% [60], but it remains expensive to produce and distribute, and its availability is limited in low- and middle-income countries [54].

The landmark multicenter, double-blind, randomized controlled trial [101] (SAFE, Saline versus Albumin Fluid Evaluation) compared 4% albumin to normal saline in the routine care of ICU patients (n = 6998). About 43% were surgical patients in either arm, with half being emergent cases. Only about 17% were pre-defined as trauma patients. Overall, there were no significant differences in outcomes between groups among all patients (including organ failure, hospital or ICU length of stay, mechanical ventilation, renal replacement therapy, or 28-day mortality from any cause). The sub-group analysis of trauma patients revealed a relative mortality risk (RR) of 1.36 with albumin compared to saline. Conversely, both the severe sepsis subgroup (RR 0.87) and ARDS subgroup (RR 0.93) appeared to have a trend towards improved survival. The authors concluded that albumin and saline should be considered "equivalent" in a heterogenous population of ICU patients. Very similar conclusions were reached in the Albumin Italian Outcome Sepsis (ALBIOS) trials where it was stated "…the use of albumin in addition to crystalloids to correct hypoalbuminemia, as compared with the use of crystalloids alone, in patients with severe sepsis during their stay in the ICU did not provide a survival benefit at 28 or 90 days, despite improvements in hemodynamic variables" [102]. Interestingly, post-hoc analysis of 63% of patients with sepsis in shock did in fact reveal a significant 90-day mortality benefit, where authors postulated albumin may be maximally exploited in those with cardiovascular dysfunction.

In a 2007 post-hoc follow-up study [103] of the subgroup of TBI patients, resuscitation with albumin was associated with a 24-month mortality RR of 1.63 (95% CI 1.17-2.26). This outcome was attributed to increased intracranial pressure from cerebral edema, particularly during the first week after injury,

despite these patients being treated with higher doses of sedatives, analgesics, and vasopressors, and received greater temperature control [104]. Findings were consistent with the theory that increased extravasation of albumin from areas of altered blood brain barrier permeability may lead to increased cerebral interstitial colloid osmotic pressure and increased ICP [105]. They suggested that albumin be avoided in patients with severe TBI, especially in the first week after trauma.

Dextrans: Dextran alone, though it increases intravascular volume, is not universally used as a standard resuscitation solution in most countries. However, recently a Swedish group published a comparison of dextran, HES and albumin in guinea pig with hemorrhagic shock and found that both plasma volume expansion and mean arterial pressure was greatest for dextran, followed by albumin [106]. Dextran use is limited by its anticoagulation effects, which allows it to be used as a weak anticoagulant in extremity ischemic vascular disease and to prime hemodialysis machines [107, 108]. Two recent Cochrane reviews of human studies, concluded that no recommendations on safety or efficacy could be made on the use of dextran-70 [109, 110].

Gelatins: Gelatin polymers are degradation products of collagen hydrolysis and are used as synthetic solutions to expand plasma volume in hypovolemic patients [111]. More recent iterations use gelatin modifications such as succinylated and urea-linked gelatin molecules [112, 113], which perform better and produce a more uniform and stable molecule in vivo. Gelofusine (B. Braum: Melsungens, DE), a 30 kDa succinylated gelatin not been approved in the US by FDA is used clinically in Europe. It is a 4% concentration and packaged in 500 and 1,000 ml Viaflex-type bags and provides an equivalent oncotic pull as human albumin in plasma. The goal in using gelatins was to overcome the equilibration effect of resuscitating low volume patients with simple saline solutions where the intravenously administered salt water rapidly equilibrates by leaving the intravascular space into the interstitial and intracellular spaces. Of course, this reduces intravascular volume, pressure, and flow, which was the main point of giving IV fluids. Large enough gelatin polymers impermeant to the capillary membrane creates oncotic effects that hold water in the intravascular space to prevent equilibration effects and third spacing, analogous to starch and dextran polymers in solution. Depending on the initial concentration gradients, gelatin solutions can also pull water into the capillary space from the interstitial space to expand the vascular volume in addition to holding volume in.

The volume expanding and hemodiluting capabilities of a 4% solution of 30 kDa succinylated gelatin (Gelofusine) are similar to 6% solutions of

hydroxyethyl starch (e.g., HES, Voluven) in both normal subjects and those undergoing surgery [114]. Gelofusine escapes into the extravascular space about one hour infusion and can cause suppression of the renin-angiotensin-aldosterone system resulting in a rapid renal volume excretion of salt and water [115, 116]. While Gelofusine increases mean arterial pressure and cardiac output it does not increase mesenteric blood flow but produces a renal glomerular hyperemia that contributes to salt and water filtration as well as some degree of albuminuria [114]. There are several reports of significant but infrequent systemic anaphylaxis reactions in patients receiving gelatins [117] occurring more frequently than with albumin and less frequently than with dextrans [118]. Gelatin based products are also associated with significant hypercoagulability. Orthopedic surgery patients showed aberrations in thromboelastography (TEG) testing after infusion of 1 liter of Gelofusine that were characterized by increases in angle, decreased R, with no change in MA [116] suggesting an increase in coagulation initiation without an effect on overall clot size or strength. In summary, gelatin based volume expanding crystalloids increase plasma volume temporarily restoring cardiac output in hypovolemic patients no superiority to saline can be demonstrated [119]. Furthermore, the use of gelatins is associated with important side effects (coagulation, anaphylactic, and renal-hormonal effects) that may offset any small benefit from a more rapid volume expansion.

New Horizons

Impermeants and Polyethylene Glycol

As discussed above crystalloid resuscitation of hemorrhage results in tissue edema resulting in the compression of local capillaries from the outside by swollen parenchymal cells and from the inside by swollen endothelial cells. This increases resistance or complete occlusion to flow, which reduces oxygen delivery to already ischemic tissues. To combat this effect, low volume crystalloid resuscitation solutions containing various cell impermeants have recently been proposed. Cell impermeants are small, stable, non-metabolizable molecules that are large enough to freely escape the capillary space but too large or too charged to cross the cell membrane. These molecules preferentially load into the interstitial space as they equilibrate across the capillary where they create an osmotic force outside the cell that pulls water out or prevents water from moving in. As swollen cells lose water, the local microcirculation decompresses and capillary flow resumes, even under very low volume states.

In pre-clinical models, the addition of impermeants like gluconate in saline increased the time allowable in the 'low volume state' prior to definitive resuscitation. This effectively doubled the "golden hour" primarily due to improved capillary blood flow from its ability to prevent cell and tissue swelling [86]. When a colloid is added to the impermeant, a double osmotic gradient is established between the cell and interstitium and between the interstitium and the capillary space, which further accelerates the non-energetic movement of isotonic water from the cell and interstitium into the capillary space. This decompresses the capillaries and increases capillary filling, thus producing an almost geometric increase in tolerance to the low volume state after low volume crystalloid resuscitation [120].

Polyethylene glycol 20,000 (PEG-20k) is a hybrid molecule that partially (30%) moves into the interstitium to act as an impermeant and partially (70%) remains inside the capillary to act as a colloid [120]. A 10% solution of PEG-20k given at low volume (10% of the estimated blood volume) increase the time that rodents can stay in the low volume state by 20 times over control using saline, Hextend, or albumin solutions [120, 121]. More recently, these PEG-20k solutions have been shown to produce similar results in pre-clinical large animal models in swine [122]. Low volume crystalloid resuscitation (LVR) solutions containing 10% PEG-20k increase survival time in the low volume state, increase mean arterial blood pressure, cause rapid clearance of lactate to normal levels after 4 hours, double cardiac output, return heart rate to control values, double splanchnic blood flow, and increase survival from zero to 100%. This potentially ideal dual mechanism of plasma volume expansion while increasing tissue capillary oxygen transfer requires further study prior to generalized use.

SUMMARY AND CONCLUSION

Crystalloid use in resuscitation from hemorrhagic shock currently have limitations that are dependent on the availability of more efficacious treatments. Specifically, crystalloids (LR and HES) should only be used in the smallest quantities possible (250-500ml boluses) to achieve life-saving outcomes of oxygen delivery when blood products are not available (measured by mental status, peripheral pulse, or targeted systolic pressures), and not at all or under limited goal-directed use when blood products are available. However, there remains in 2016 controversy as to which crystalloid type is best. Military

(Tactical Combat Casualty Care) and German [S3] guidelines recommend HES, while civilian U.S. (Prehospital Trauma Life Support) guidelines recommend LR. Practically, crystalloid use will continue in pre-hospital settings where blood products or alternatives are not currently available, and likely, even when they are. Their use should be restricted to low volume resuscitation with permissive hypotension to augment safe transport and evacuation. Advances in newer IV agents that greatly enhance the efficiency of capillary exchange, which happen to be crystalloid, may expand the role of future crystalloid use, especially if combined with stable transportable blood products like lyophilized or spray dried plasma components. These newer agents may also see use in hospital and ICU settings as adjuncts to blood products in the future.

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PLOS ONE

RESEARCH ARTICLE

Thromboelastographic analysis of novel polyethylene glycol based low volume resuscitation solutions

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Abstract

Background

Low volume resuscitation (LVR) in shock prevents deleterious effects of crystalloid loading in pre-hospital settings. Polyethylene glycol 20,000 (PEG-20k) based LVR solutions are 20-fold more effective at maintaining perfusion and survival in shock compared to conventional crystalloids. The aim of this study was to determine coagulation and platelet function of whole blood treated with 10% PEG-20k.

Methods

Citrated blood from volunteers (n = 25) or early admission severely injured trauma patients (n = 9) were diluted 10% with various LVR solutions in a matched design with a paired volume control (saline), and studied using thromboelastography (TEG).

Findings

In healthy volunteers and patients, 10% PEG-20k significantly increased clot amplification time (k), decreased propagation (angle), maximal clot size and strength (MA), and the overall coagulation index (CI), but not clot initiation (R) or fibrinolysis (Ly30), relative to paired saline dilutional controls. Clinically, K, angle, and MA were just outside of the normal limits in volunteers but not in patients. No statistical differences existed between PEG-20k and Hextend (HES) in either patient population. In a dose response series using volunteer blood, all effects of 10% PEG-20k on TEG were reversed and normalized by lower concentrations (7.5% and 5%). Furthermore, 7.5% PEG-20k produced similar resuscitation effects as 10% PEG in rodent hemorrhagic shock models (n = 5).

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Abbreviations: 0.9% NaCl, NS, Normal saline; HES, Hydroxyethyl Starch; HS, HEXTEND; IRB, Institutional Review Board; ISS, Injury severity score; JTTS CPG, Joint Theater Trauma Systems Clinical Practice Guidelines; LR, Lactated Ringer's; LVR, Low volume resuscitation; PEG-20k, Polyethylene glycol (PEG) 20,000 mw; TEG, Thromboelastography; TIC, Trauma induced coagulopathy; TICS, Trauma-induced capillary leak syndrome; VCU, Virginia Commonwealth University; WB, Whole Blood, undiluted.

Conclusions

In conclusion, PEG-20k based LVR solutions produced a dose-dependent minor hypocoagulative state, possibly associated with changes in clot propagation and platelet function, which can be reversed by dose reduction in concentration while providing superior LVR, microvascular rescue, and lactate clearance compared to saline or starch.

Introduction

Minimizing the use of crystalloids and utilizing blood products after trauma are now becoming mainstream in civilian trauma centers. Damage control resuscitation is also emerging as standard of care for the US Department of Defense, according to the Joint Theater Trauma Systems Clinical Practice Guidelines (JTTS CPG). When blood products are not available for resuscitation, crystalloid solutions are administered. However, only a fraction of infused conventional crystalloid volume stays in the intravascular space so the use of low volume crystalloids has minimal effects on pressure and perfusion [1, 2]. The movement of crystalloid fluid from capillary to interstitium is compounded by the increase in capillary permeability from trauma-related inflammation and trauma-induced capillary leak syndrome (TICS) [3]. Furthermore, crystalloid resuscitation exacerbates TICS, acidosis, hypothermia, and coagulopathy [3, 4]. Other resuscitation solutions such as hypertonic saline or starch have had disappointing results [5, 6] including concerns and risks associated with their use [4, 7]. There remains a need for a better crystalloid fluid that can be given at a low volume to resuscitate patients in severe hemorrhagic shock awaiting definitive treatment, especially for the prehospital setting.

Recently, polyethylene glycol (PEG) polymers of specific molecular weight ranges have been used in crystalloid solutions to act as highly effective Low volume Resuscitation (LVR) solutions [2, 8–10]. These polymers non-energetically move isotonic fluid from intracellular and interstitial spaces into the capillary space by osmotic flow mechanics in response to metabolic cell swelling that occurs in shocked and ischemic tissues. During low-oxygen states, energy-dependent ion homeostasis break down and membrane function becomes dysregulated, allowing an imbalance of solute and solvent within these spaces. Cellular swelling, apoptosis, necrosis, and a loss of capillary flow dynamics perpetuates the cycle of shock [4, 7]. Reversing and preventing these effects may be possible with protective resuscitation solutions containing polyethylene glycol (PEG) that alters fluid volume transfer in cell and extracellular spaces during shock.

PEG-20k molecules are impermeant to cells and selectively partition in the capillary and interstitial spaces in a ratio of 2:1, respectively [2, 10]. This pulls isotonic fluid out of swollen cells and tissues. As water flow moves from the interstitial spaces to the capillaries, the capillary exchange in the tissues dramatically improves under very low volume conditions because the microcirculation is decompressed, lowering the resistance to flow, while the capillary spaces are re-loaded with volume and pressure for driving flow [10]. This causes rapid clearance of lactate, increased blood pressure, and tolerance to the low volume state [8]. Thus, despite being in a post-hemorrhagic state under very low volume conditions with dysregulated membrane mechanisms, polyethylene glycol was able to decompress the microcirculation to improve capillary exchange in the tissues and dramatically optimize resuscitation. Interestingly, the polyethylene glycol polymers were shown to work several fold better than hydroxyethyl starch based solutions in these hemorrhagic resuscitation studies, implying different mechanisms of action of the polymers, despite theoretically similar mechanisms of exerting

oncotic pressures intravascularly. Differences in underlying biochemical properties, size, and structure are likely to play a large role in these differences by causing them to partition differently in the microcirculation and pull water at different rates. Additional differences may also include vascular interactions with the glycocalyx and capillary structures in varying shock and ischemic states or the formation of hydration shells [11, 12].

Intravenous administration of Hextend (hydroxyethyl starches) have well known complications of both renal toxicity and coagulopathies [13], which in trauma or acute care settings are definite concerns, especially given the expanding literature on trauma related coagulopathy or shock induced endotheliopathy [13]. Interference with blood clotting and coagulation may be shared by both PEG and starch polymers, due to hemodilution or other cell-mediated causes, despite the aforementioned differences. Therefore, the purpose of this study was to examine any possible effects of PEG-20k based LVR solutions on overall whole blood coagulation and platelet function in both healthy volunteers and a selection of trauma patients using thromboelastography.

Methods

Low volume resuscitation (LVR) solutions

Normal saline (0.9% NaCl, NS; prepared using 9 g/L sodium chloride) served as both a crystalloid vehicle volume control (as all solutions were prepared in a normal saline base), and as a dilutional volume control (as all blood samples were diluted 10% with each solution, to represent the 10% volume administered after hemorrhagic shock in all previous rodent and porcine LVR studies) [1, 2]. A military medicine resuscitation comparative control used a 6% hydroxyethyl starch (HES) solution and was purchased from the manufacturer under product name HEXTEND (HS), formulated with 6% hetastarch [molecular weight (MW) ~600 kDa (range 450–800 kDa) with ~0.75 molar substitution at primarily the C-2 glucose unit] in 0.9% sodium chloride. The experimental solutions consisted of polyethylene glycol (PEG) 20,000 mw (PEG-20k) dissolved in normal saline at concentrations of 10%, 7.5%, and 5%. Polyethylene Glycol-20k was purchased from Sigma Chemical Co (St. Louis, MO) as the molecular biology grade material. All solutions were either prepared fresh or filter sterilized using 0.22 micron filtration for storage in polypropylene containers to exclude bacterial or polymer degradation.

Preparation of blood and TEG assay

An internally matched comparative analysis was designed where each study participant would serve as their own control. Each enrolled study participant's blood was diluted 10% with each studied resuscitation fluid (NS, HS, PEG), always including a saline (NS) dilution control paired with 6% HES, and 10% PEG-20k. A dose-response series was also conducted using PEG-20k at concentrations of 5%, 7.5%, and 10% in saline at the same 10% dilution with whole blood. Both healthy volunteers and trauma patients were enrolled and consented under an approved Virginia Commonwealth University (VCU) IRB protocol. Healthy volunteers were without comorbidities or any medications and between 18–50 years of age. Trauma patients were those of the same age arriving at the highest alert level to our trauma system with any injury or mechanism, as long as they had evidence of severe hypovolemia or ischemia represented by a systolic blood pressure below 95 mm Hg, a plasma lactate level $\geq$ 4.6 mM, or an injury severity score (ISS) greater than 24. Blunt and penetrating trauma were included. Trauma patient blood was collected early after arrival, usually within 30 minutes and prior to any blood transfusions or significant crystalloid infusions. The goal was to select only those patients that received only small amounts (0–300 ml) of saline or Lactated Ringer's (LR)

crystalloid in the field or en-route to the emergency department. Patients who received larger fluid volumes or blood products were excluded from the study.

Venous blood samples from individual healthy volunteers or from trauma patients were drawn into citrate treated vacutainer tubes (15 ml total), pooled, and diluted 10% with saline, 6% Hextend, 10% PEG-20k, 7.5% PEG-20k, or 5% PEG-20k. Prior written consent was obtained from the volunteers and after the fact written consent was obtained from the trauma patients or their legal advocate. In cases where consent was either denied or not obtained after the fact from the patients, the blood samples and any results obtained from them were destroyed. Some TEGs were run on undiluted whole blood. They were then gently mixed by inversion, and analyzed on a TEG-5000 thromboelastograph (Haemonetics Corp.) within 2 hours from blood draw using kaolin activation by trained laboratory staff on machines calibrated daily. Each blood sample was analyzed by TEG twice and the values averaged. Each unique patient or volunteer blood sample was the source for all dilutions and the experimental values were compared to the same blood either undiluted or diluted equally with the vehicle (saline). Therefore, each sample served as its own control, which would take into account any variations that might be present in the blood sample hematocrits, platelet counts, or plasma protein samples (such as fibrinogen). The TEG data were reported as six outcome parameters that describe different functional attributes of the clotting and coagulation system of whole blood under these conditions. These include: **R**, a measure of the time to initiate fibrin clot formation; **k**, time to achieve a predetermined clot size and strength (20 mm clot size). This represents amplification of the clotting cascade; **Alpha (α)** or angle of the slope between R and k, which characterizes the propagation phase and thrombin burst converting fibrinogen into fibrin with fibrin cross linking; **MA**, the maximum amplitude of the clot that represents clot strength, which is generally composed of 80% platelet and 20% fibrin responses; **LY30**, the % lysis of the clot 30 min after maximal formation (MA), which represents rates of fibrinolysis of the clot; and **CI**, the coagulation index that is a mathematical model of overall coagulation responses using the other TEG parameters.

Shock resuscitation testing

To test resuscitation outcomes of LVR solutions specifically for comparison with TEG outcomes in the dose response series of experiments, a standard rat lactate controlled model of severe hemorrhagic shock with low volume resuscitation was used as previously described in great detail elsewhere [2, 9, 10]. These studies were approved by the VCU IACUC and followed the ARRIVE guidelines [14]. Briefly, we determined tolerance to the low volume state in severely shocked acutely anesthetized rats (n = 5 for each group). Arterial bleeding to a mean arterial pressure of 35 mmHg was maintained until plasma lactate rose to 9–10 mM, which initiated low volume resuscitation using saline control, or 10% and 7.5% PEG-20k solution, all given intravenously at a volume equal to 10% of the estimated blood volume of the rat [15]. Immediately after LVR solutions are given, lactate falls but then begins to rise again until it again reaches the 9–10 mM limit. The time from the start of LVR infusion until the lactate rises back to its limit again (9–10 mM) is recorded as the LVR time. The LVR time and the lactate and MAP values at the end of the LVR time are all outcome measures of the tolerance to the hypotensive state.

Statistical analysis

All statistical analyses were performing using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Data groups were analyzed for outliers using the nonlinear regression ROUT method with Q = 1%, the maximum

desired false discovery rate. Normality of Gaussian distribution was then assessed using the D'Agnostino-Pearson ombinus K2 method. Most data were then analyzed by the non-parametric ANOVA Kruskal-Wallis test with the Mann-Whitney U test for multiple comparisons of means. Most TEG data are expressed as the median with the 2nd and 3rd interquartile ranges and the upper and lower extremes (box and whiskers). Significant differences from clinical limits of normal for laboratory values were determined using the Wilcoxon Signed Rank Test. Population data is expressed in mean ± SD.

Results

Solutions

All solutions were prepared with a base of NaCl that gave a final concentration of Na = 154 mEq and Cl– 154 mEq. The final solution calculated osmolarity of the three solutions that were used in most of these studies were; Saline vehicle control = 308 mOsm, 6% Hextend = 308.1 mOsm, and 10% polyethylene glycol 20k = 308.5 mOsm. The calculated osmotic pressure of each component was 15.06 Atm for the NaCl vehicle component, 0.122 Atm for the PEG-20k component in the PEG-20k solution, and 0.0027 Atm for the hydroxyethyl starch component of the Hextend solution (assuming an average molecular weight of the HES of 550,000 Da).

Populations

The healthy volunteer population (n = 25) was enrolled intermittently between 7/2015-7/2017. Ages ranged 20–45 (28.4 ± 6.21), and 14/25 (56%) were males. Of note, HES colloidal comparative controls had n = 7 sample. The dose response group for PEG concentrations had n = 9 sample. All PEG or HS samples were matched with saline control. The trauma patient population (n = 9) was enrolled intermittently between 10/2016 and 5/2017. Ages ranged 18–39 (29 ± 8.8) years, and 7/9 were males. Penetrating injuries with or without polytrauma were seen in 3/9 patients (due to gunshot wounds to the trunk), while 6/9 patients presented with blunt/polytrauma injuries (due to motor vehicle or motorcycle collisions, or a 40ft fall in one case). Injuries were widespread including visceral lacerations (5/9), orthopedic fractures (5/9), hemo/pneumothorax or pulmonary contusions (5/9), burn (1/9), and traumatic brain injuries (4/9). Lowest pre-hospital or ER systolic blood pressure (SBP) ranged 50–124 (90.8 ± 1.56, n = 9) mmHg, while diastolic BP ranged 24–82 (60.8 ± 1.56, n = 9). Plasma lactate ranged 1.1–5.3 (3.3 ± 1.56, n = 5) mmol/L. ISS ranged 9–48 (30.2 ± 13.6, n = 9). The main purpose of the trauma patient group was to examine how their blood responded to PEG-20k LVR solutions and not necessarily to directly compare them to the volunteers or to show a trauma-induced coagulopathy.

Initiation of clotting

The TEG R time is shown in Fig 1. For healthy volunteers using 10% diluted whole blood, mean initiation times were lower than the normal range for both saline and HES diluted whole blood, significantly so for the saline, while being just within normal limits in PEG diluted blood. PEG significantly lengthened the initiation time, relative to the saline dilutional controls. Similar trends were seen in both diluted and whole blood from trauma patients, where all of the R times were below normal. For PEG dose responses, all values were below normal irrespective of PEG concentration ranges from 5–10%. The volunteers and patients showed similar R times for all groups. All normal TEG values were reported from Haemoinetics, Inc.

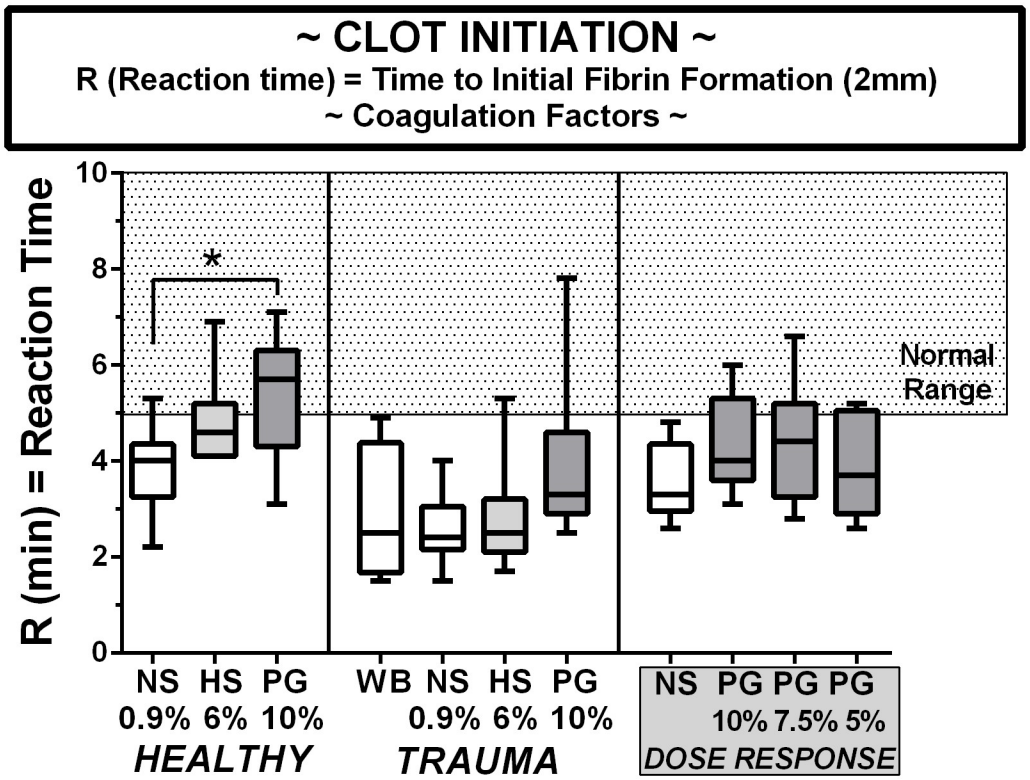

Fig 1. Clot initiation indexed by the R time on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25 healthy volunteers, 9 trauma patients, and 9 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PEG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2–3 hours of blood draw in a matched design with saline always serving as control to the resuscitative fluid. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB = whole blood (undiluted). *$P<0.05$.

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Amplification of clotting

The TEG k parameter is shown in Fig 2 for the volunteers, the trauma patients, and a PEG dose response series of diluted blood. The trend in all groups was a significant increase in the amplification time in both HES and PEG diluted whole blood compared to saline diluted blood. There were no differences in the whole blood and saline-diluted blood in the trauma group, for any parameter. While there was a significant difference between normal saline and both the 10% and 7.5% PEG dilutions within the healthy volunteer dose-response group, the elevated K time was returned to the normal range for both 7.5% and 5% solutions in a dose-dependent manner, simply by decreasing the concentration of PEG in the LVR solution.

Clot propagation

Fig 3 shows the data for the TEG angle parameter in volunteers, trauma patients, and healthy volunteer blood in the PEG dose-response study. The angle substantially decreases in both HES and PEG diluted blood compared to saline, with significant decreases in PEG groups. This effect is qualitatively and quantitatively the same in blood obtained from both volunteers

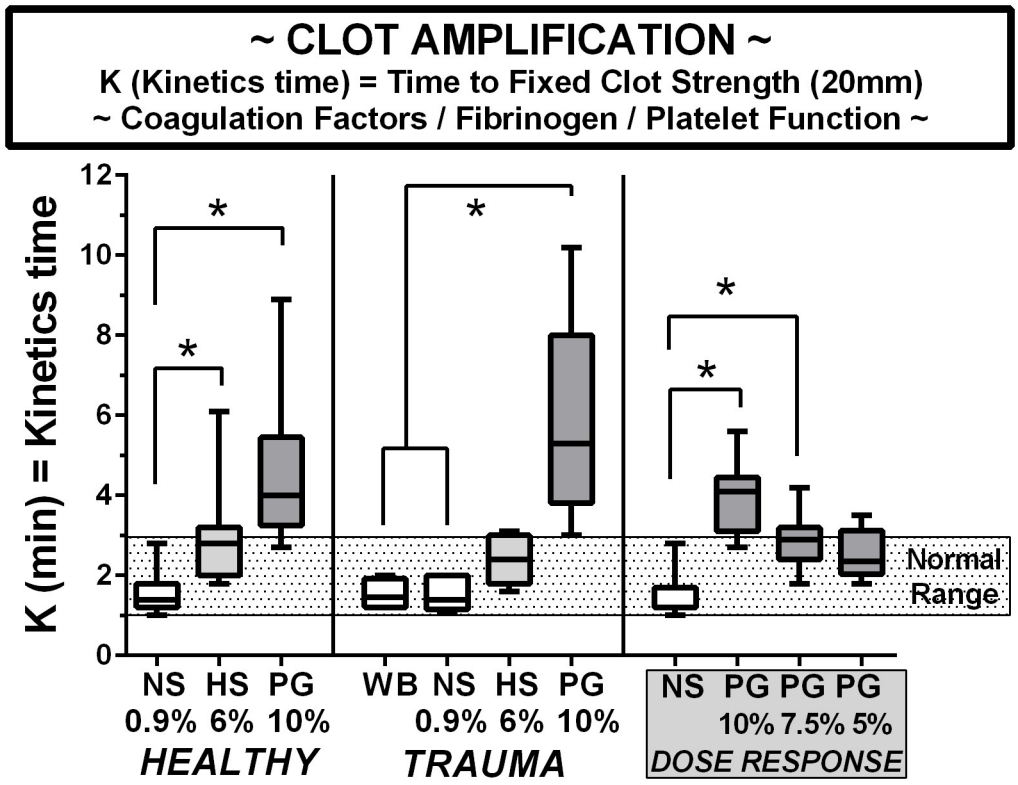

Fig 2. Clot amplification indexed by the k time on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25 healthy volunteers, 9 trauma patients, and 9 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2–3 hours of blood draw in a matched design with saline always serving as control to the resuscitative fluid. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB = whole blood (undiluted). *$P<0.05$.

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and trauma patients. The significant decrease in the angle or propagation rate by 10% PEG-20k was normalized by reducing the concentration to 7.5% and 5% in the LVR solutions, seen in the dose-response section, similar to k.

Maximum clot strength

The TEG MA parameter is shown in Fig 4 for blood from healthy volunteers, in blood from trauma patients, and volunteer blood in a PEG-20k dose-response series. The maximum strength or clot size is generally believed to represent a contribution by both platelets (80%) and fibrin (20%) under these conditions. While, the MA response is reduced in both HES and PEG diluted blood, significantly so in the 10% PEG-20k group relative to the saline dilutional controls, the effect is less severe than seen with either parameters k or angle, given the proximity of the means to the outer limits of normal range and absolute difference between means of the groups. The patterns again are almost identical in blood obtained from both healthy volunteers and trauma patients, as no significant differences exist between the groups. The significantly lower clot strength in blood diluted with 10% PEG-20k could again be dose-

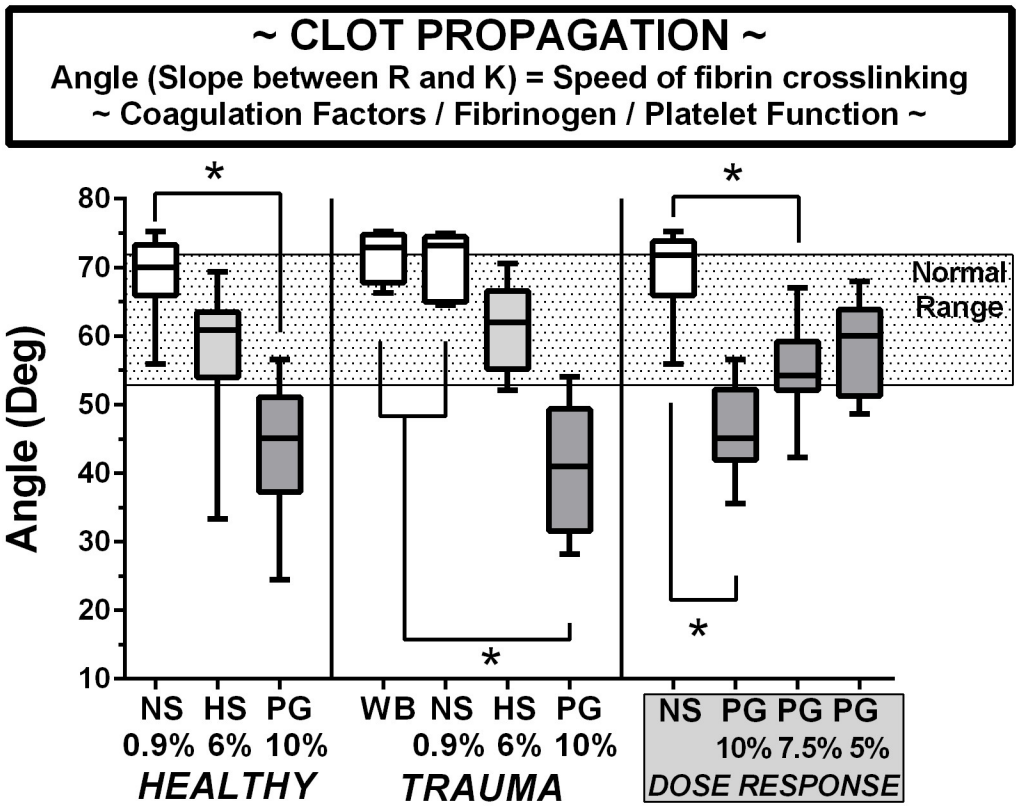

Fig 3. Clot propagation indexed by the Angle variable on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25 healthy volunteers, 9 trauma patients, and 9 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2–3 hours of blood draw in a matched design with saline always serving as control to the resuscitative fluid. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB = whole blood (undiluted). *P<0.05.

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dependently reversed by progressively lowering the PEG concentration in the LVR solutions to 7.5% and 5%.

Clot lysis

The TEG Ly30 data are provided in Fig 5. The rate of clot lysis was mostly less than 1–2% after 30 minutes and was not affected by the dilution with any LVR solution, including PEG-20k. The rate of fibrinolysis was also not different in the trauma patients compared to healthy volunteers. As opposed to the other TEG parameters, NS and WB groups had much larger ranges than the HS or PG counterparts. Additionally, there appears to be a nonsignificant but slightly notable trend of higher concentration PEG to dampen any fibrinolytic response measured under these ex-vivo settings.

Coagulation index

The coagulation index is shown in Fig 6 for the blood dilutions in the healthy volunteers, the trauma patients, and the volunteers in the PEG-20k dose-response study. The coagulation

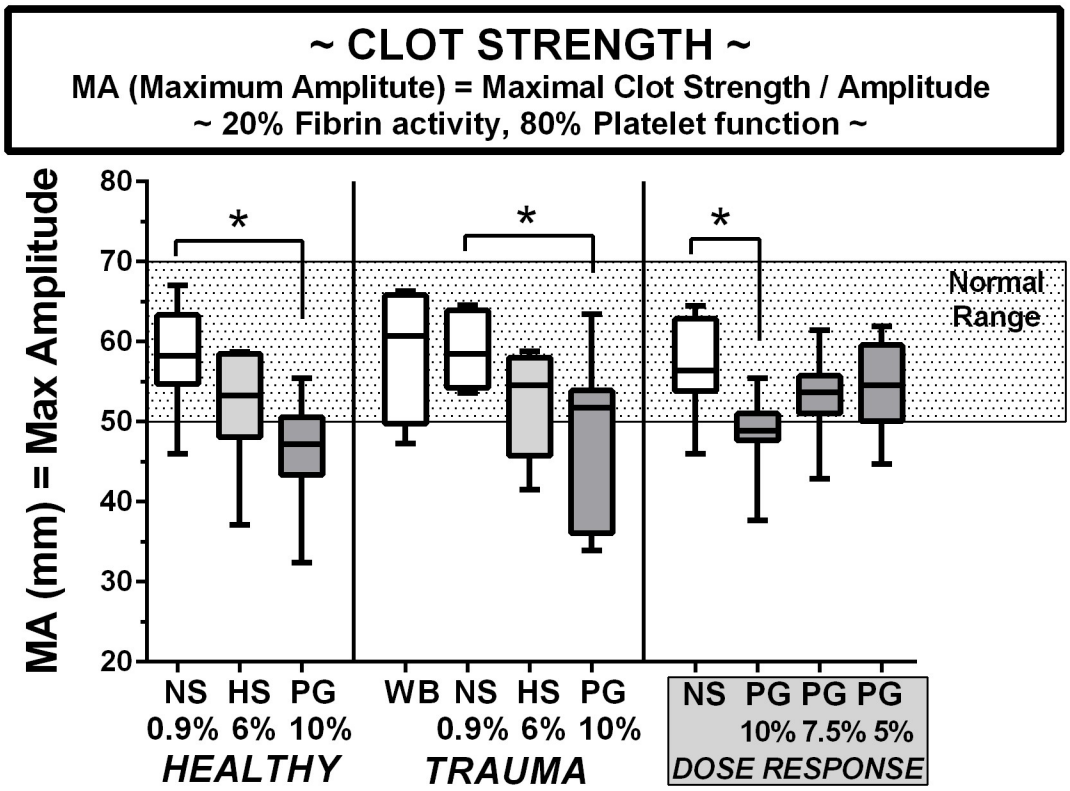

Fig 4. Clot strength and maximum size indexed by the MA on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25 healthy volunteers, 9 trauma patients, and 9 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2–3 hours of blood draw in a matched design with saline always serving as control to the resuscitative fluid. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB = whole blood (undiluted). *P<0.05.

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index (CI) is a mathematical compilation of other TEG parameters and is described by CI = -0.3258R-0.1886K+0.1224MA+0.0759α-7.7922. The normal range for CI is between 3.0 and -3.0. As shown in Fig 6, blood from either healthy volunteers or trauma patients had substantial reduction in the CI with HES and significant reduction with PEG-20k relative to saline control, similar to trends discussed in other parameters. However, there is NO statistical difference from lower limit of normal for any group. Of note, to the left of the CI panel are representative TEG tracings to indicate increasing level of hypocoagulability with narrowing tracing. The CI could be dose dependently reversed into the normal range by reductions in the concentration of the PEG-20k in the LVR solution.

Resuscitation performance of 7.5% PEG-20k solutions

Because slight reductions in the concentration of PEG-20k in LVR solutions (to 7.5% or 5%) caused a normalization of TEG parameters relative to those observed using 10% PEG, the effects of the reduced concentration on resuscitation outcomes was determined in our common rodent hemorrhagic shock model. Six Sprague Dawley rats were used for each group but one was excluded in the 7.5% PEG-20k group (total n = 14). Fig 7 shows that reducing the

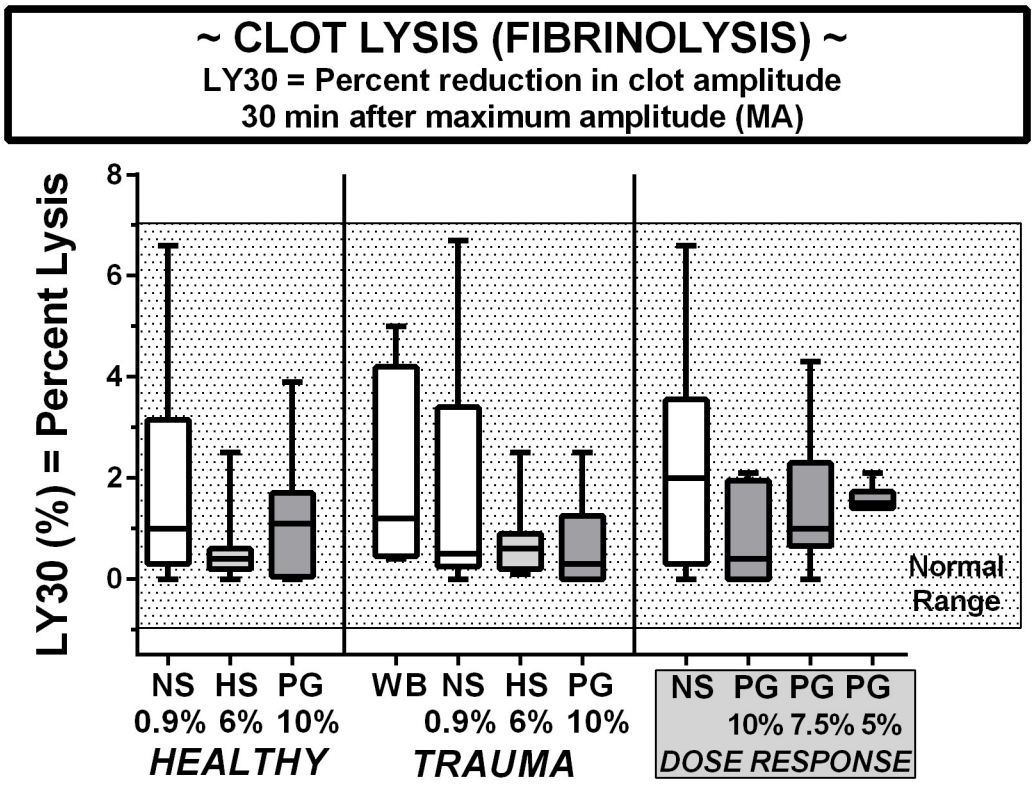

Fig 5. Clot lysis indexed by the LY30 on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25 healthy volunteers, 9 trauma patients, and 9 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2–3 hours of blood draw in a matched design with saline always serving as control to the resuscitative fluid. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB = whole blood (undiluted).

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concentration of PEG-20k from 10% to 7.5% produces an equivalent resuscitation effect to the one observed with 10% PEG-20k. This is true when the quality of the resuscitation is described by the LVR time (panel A), the terminal lactate values (panel B), or the terminal mean arterial blood pressures (MAP, panel C). LVR time (i.e. tolerance to the low volume state) of either PEG-20k solution (10% or 7.5%) is approximately six times that of saline (40 ± 20.3 vs. 240 ± 6.7 minutes), although true LVR time is unknown as experiments were stopped at 240 minutes. End lactate of 7.5% PEG (2.9 ± 1.5 mmol/L) is approximately a quarter of saline (10.6 ± 2 mmol/L), while 10% PEG is approximately one eighth compared to saline (1.2 ± 0.13 mmol/L). MAPs are also approximately 2.5 times higher with PEG solutions (67.8 ± 7.2 and 81.6 ± 17.6 mmHg for PEG 7.5% and 10%, respectively), vs saline (30.9 ± 7.2 mmHg).

Discussion

Low volume resuscitation and polyethylene Glycol-20k

Low volume crystalloid resuscitation is used in early pre-hospital resuscitation of severely shocked patients in civilian and military settings for two important reasons. First because

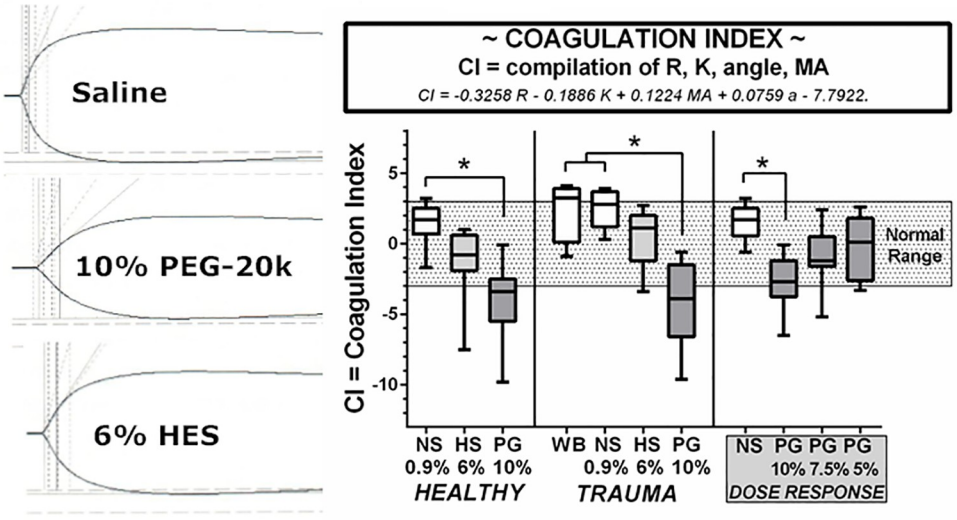

Fig 6. Coagulation Index as measured by the CI on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. The CI is a mathematical compilation of other TEG outcome variables (R, K, Angle, and MA). All TEG outcomes represents 25 healthy volunteers, 9 trauma patients, and 9 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2–3 hours of blood draw in a matched design with saline always serving as control to the resuscitative fluid. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB = whole blood (undiluted). Tracings on the left are representative TEG tracings obtained from all three LVR solutions used in this study (NS, PEG-20k, and HES). *P<0.05.

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lower dilutional volume replacement has superior outcomes compared to the traditional large volume resuscitation strategies [13] and second, because low volume crystalloid is friendly to resource poor austere environments of distant field locations, especially in forward military theatres and geographically challenging regions.

Therefore, a new crystalloid low volume resuscitation solution has been developed and tested in pre-clinical hemorrhagic and trauma shock models and found to be highly effective

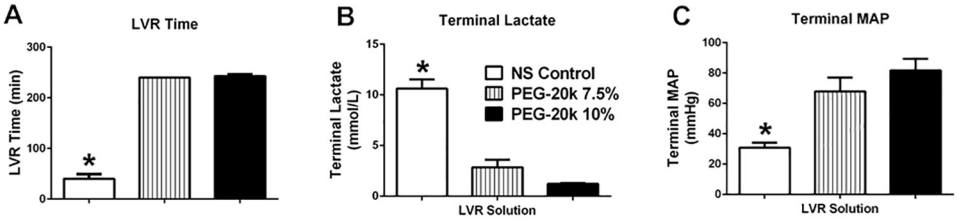

Fig 7. Acute resuscitation outcomes in a study in rats subjected to severe hemorrhagic shock and low volume resuscitation with NS (saline) controls, or 7.5% and 10% PEG-20k. All LVR solutions were given at a volume equal to 10% of the estimated blood volume of the animals. The low volume resuscitation times (LVR) were measured and shown in panel A, which is an index of tolerance to the low volume state (see Methods for details). The end or terminal lactate values are shown in panel B, which are the values at the end of the LVR period (or 240 minutes in the PEG groups), and the terminal mean arterial blood pressure measured at the end of the LVR period in panel C. Values are mean +/- standard deviation, n = 5 for NS and 10% PEG-20k and n = 4 for 7.5% PEG-20k. Baseline MAP before hemorrhage was 101 +/- 18 mmHg and baseline lactate before hemorrhage was 0.9 +/- 0.2 mM/L, *P<0.05 relative to both PEG groups.

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in increasing the tolerance to the low volume state by significantly increasing microcirculatory oxygen transfer efficiency. These solutions are based on specifically sized polymers of polyethylene glycol solutes (PEG-20k) that work by osmotic and hydrophilic actions in the microcirculation. These forces non-energetically move isotonic fluid out of metabolically swollen cells into capillaries thereby reloading the exchange vessels and propelling convective oxygen transfer by decreasing the resistance to flow via their primary effects on cell and tissue swelling (tissue decompression). The result is rapid oxygen debt repayment, lactate clearance, and re-establishment of oxygen transfer under very low volume conditions.

This approach is ideal for pre-hospital use because metabolic and cardiovascular tolerance to trauma increases, which can safely lengthen evacuation and transport times and ensures better outcomes when definitive resuscitation occurs at a civilian or forward military hospital [8]. Since the active molecules in these new solutions are large polymers not unlike hydroxyethyl starch (HES) and because they produce significant water transfer and dilutional effects in blood compartments, the effects of these solutions on whole blood coagulation and platelet function are of possible concern and are as yet unknown. Therefore, the purpose of this study was to characterize the effects of LVR solutions containing PEG-20k on whole blood coagulation and platelet function using TEG analysis (this report) and on more detailed mechanisms of coagulation and platelet function using more specific testing in a companion paper to this one.

Extrapolating ex-vivo results to in-vivo responses

In these experiments using ex-vivo diluted whole blood, 10% PEG-20k produces a clinically recognizable coagulopathy (i.e. requiring blood product transfusion per ACS TQIP Massive Transfusion in Trauma Best Practice Guidelines) that is statistically different from normal saline dilutional control at a 10% volume dilution, but *not* from 6% HES colloidal controls in healthy volunteers or trauma patients. Additionally, it appears the effects at 10% PEG may be attenuated with lower concentrations.

The dilution factor of 10% was chosen since this is the upper limit of low volume resuscitation ranges that may be used in the field, corresponding to an approximate volume of 500 ml in an adult (with a blood volume of 5 liters). Since these studies are diluted ex-vivo, we assume the coagulation effects observed are true for patients when they are diluted at a similar 10% volume. However, in trauma patients in the field requiring low volume resuscitation, there is no way of accurately estimating if a theoretical 10% LVR dilution with crystalloid actually represents 10% or something larger or smaller. Understanding this is essential in order to extrapolate the coagulation results from this ex-vivo study that used an exact 10% dilution with the LVR solutions being tested.

The *forces favoring a greater dilution in the trauma patient* over the 10% estimated theoretical value (resulting in a lower % PEG-20k concentration) include dilution in the vascular space from subsequent movements of isotonic water from the intracellular and interstitial spaces into the capillary space, which is where coagulation and platelet function occurs. This is probably a significant dilution and can represent a doubling of the intravascular isotonic water volume that cuts hemoglobin and albumin marker concentrations in half [8, 10]. Essentially, an auto-infusion with the body's own isotonic fluid being driven by the PEG low volume resuscitation.

An additional factor that would favor a dilution of the PEG exceeding the theoretical calculated 10% after administration to trauma patients include the capillary reflection coefficient of the solute (PEG-20k). The osmotic reflection coefficient σ_d is a measure of the % partitioning of a large solute molecule (like PEG-20k) between the capillary and the extracapillary space. A molecule that has a reflection coefficient of 1.0 demonstrates 100% reflection by pores in the capillary so 100% stays in the capillary available for interactions with coagulation factors and

platelet interactions. A coefficient of 0 indicates no reflection and the solute is equilibrated equally between the capillary and interstitial spaces, or, 50% of the material and the osmotic effect is lost. The actual σ_d of PEG-20k is 0.5 in most capillary beds [2, 10], which means that 33% of the material administered into the vascular space (10% theoretically) quickly equilibrates outside of the capillary into the interstitial space. In fact, this intermediate reflection coefficient, which is rare, was a sought-after molecular attribute for choosing an ideal impermeant solute to construct an LVR solution with maximum water transfer properties. This means that, all things being equal, administration of a 10% PEG-20k solution will result in a 6.6% solution after the solute molecules equilibrate across the capillary membrane, based on the properties of the capillary as defined by PEG-20k's unique reflection coefficient. This property, along with the large pull of water into the capillary space from the osmotic and hydrophilic forces of the PEG-20k, tends to dilute out the PEG-20k concentration in the blood and reduce the effects of the PEG polymers on interference with coagulation.

The *factors that tend to increase the concentration* of PEG-20k from the theoretical 10% dilution after administration to trauma patients is the large hemorrhage volumes that are not taken into account when the theoretical blood volume is calculated. If 25% of the blood volume is lost in a trauma patient to hemorrhage, then administration of a 10% solution at an estimated 10% blood volume dilution will result in an underestimation of the blood volume and a more concentrated final PEG-20k concentration in the vascular space (to about 12.5%). This may tend to exacerbate any dose-dependent PEG-20k effects on the coagulation and platelet system. The final dilution of a 10% PEG-20k solution given at a theoretical 10% dilution in a trauma patient will be an algebraic sum of all of these forces acting together. Our preliminary modeling of a patient with a 40% hemorrhage volume suggest a dilution greater than 10%, which would lessen the coagulation side effects that had been documented here in ex-vivo whole patient blood at an exact 10% dilution. This has been validated with some preliminary animal hemorrhage studies using labeled PEG-20k. Finally, we know that we can move the PEG-20k dilution down to 7.5% and be both effective in shock resuscitation (rodent study) and neutral with respect to changing TEG parameters of coagulation and platelet function.

Proposed coagulopathy mechanisms

The mechanism of the coagulopathic effects of PEG-20k LVR solutions on clotting blood cannot be determined from the TEG data because the tests are descriptive. However, certain mechanistic effects can be inferred from the individual changes in the TEG outcome variables. The TEG responses in both healthy volunteer and trauma patient blood diluted 10% with PEG-20k solutions (at 10% concentration) suggests mainly an interference with direct platelet function (because MA is reduced) and possibly indirect effects of the platelet contribution to coagulation from thrombin generation (because k and angle are affected). However, direct platelet aggregation tests were not performed in this study so a detailed analysis of this contribution to our TEG results is not possible. The R value was slightly elevated suggesting a mild enzymatic initiation defect too. Other possible effects on coagulation reactions cannot be excluded from the TEG data alone.

Proposed clinical utility

The effects of PEG-20k on coagulation and platelet function, as assessed by TEG, were the same in blood from health volunteers and trauma patients, despite widely disparate presumed baseline physiology during a shock state. This is good to know since our intent is to understand how these solutions influence systems in the trauma patient. While this study is limited by the ex-vivo format, that is, lacking the endothelial aspect of coagulation in real time, or the

physiologic changes of shock states, the matched controls in an ex-vivo setting allows for controlled evaluation of any baseline effects of PEG opposed to military colloid (HES) or civilian crystalloid (NS) controls.

Our selection criteria for trauma patients was strict in that we wanted severely injured patients with an Injury Severity Score (ISS) over 24, a lactate on arrival of $\geq$ 4.6 mM, or hypotension characterized by a systolic blood pressure <95 mmHg. Another goal was to measure patients as soon as they entered the trauma system because they would have a greater chance of not being transfused with blood or given significant volumes of crystalloids that would further complicate an already chaotic system. We selected a small but diverse population including multiple injury mechanisms and outcomes, with ISS averaging 30 (range 9–48) and SBPs averaging 91 mm Hg (range 50–124). Lactate was not as impressive, but did average 3.3 mM with range 1–5 mM. Given the severity of illness, it was surprising that we didn't see evidence of a trauma induced coagulopathy (TIC) in our trauma patient population, especially a temporary hyper-coagulative state. We also did not follow these patients in time to document the development of a later TIC or hypocoagulative state. In any case, the effects of PEG-20k LVR solutions behaved almost identically in patients as it did in healthy volunteers.

Conclusions

This study compared various concentrations of PEG-20k on coagulation and platelet function using TEG compared to a dilutional saline control and a clinical (military medicine) control using a 6% solution of hydroxyethyl starch (Hextend). While both Hextend and PEG-20k solutions produced measureable and significant effects on TEG outcomes, the PEG effects were not significantly different from the Hextend effects even though the absolute changes appeared more pronounced in the 10% PEG-20k groups. The commonality between PEG-20k and HES are that they both are polymers and they both have hypocoagulative effects on whole blood TEG testing. However, these polymers are chemically different and the similarity or differences in their mechanisms of action on the coagulation and platelet activation system should be speculated with caution until more definitive mechanistic testing is performed. At this point, it appears that the qualitative effects on TEG for both polymers are very similar when comparing 6% HES with 10% PEG-20k.

In conclusion, this study clearly shows that LVR solutions used for the resuscitation of patients in severe hypovolemic shock has statistically significant but minor effects on whole blood coagulation and platelet function as determined by TEG in an ex-vivo test system. The effects are not due to volume dilution and are similar to those seen with 6% HES. The PEG-20k effects are dose dependent and are essentially abrogated and reversed by reducing the PEG-20k concentrations from 10% to 7.5%. The exact mechanisms of the polymer effects on TEG are not known, but the TEG analysis suggests that platelet function or fibrinogen conversion may be involved. The clinical effects will need to be verified in an in-vivo model.

Supporting information

S1 File. Original data files and statistical analysis for all figures presented in Prism 6.0 format.
(PZF)

Acknowledgments

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Writing – review & editing: Loren K. Liebrecht, Jason Newton, Erika J. Martin, Nina Wickramaratne, Sudha Jayaraman, Jinfeng Han, Michel Aboutanos, Donald F. Brophy, Martin J. Mangino.

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Effects of Polyethylene Glycol-20k on Postresuscitation Myocardial and Cerebral Function in a Rat Model of Cardiopulmonary Resuscitation

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Objectives: Polyethylene glycol-20k is a hybrid cell impermeant that reduces ischemia injury and improves microcirculatory flow during and following low flow states through nonenergy-dependent water transfer in the microcirculation. We investigated the effects of polyethylene glycol-20k on postresuscitation microcirculation, myocardial and cerebral function, and duration of survival in a rat model of cardiopulmonary resuscitation.

Design: Ventricular fibrillation was induced in 20 male Sprague Dawley rats and untreated for 6 minutes. Animals were randomized into two groups ($n = 10$ for each group): polyethylene glycol-20k and control. Polyethylene glycol-20k (10% solution in saline, 10% estimated blood volume) and vehicle (saline) were administered at the beginning of cardiopulmonary resuscitation by continuous IV infusion. Resuscitation was attempted after 8 minutes of cardiopulmonary resuscitation.

Setting: University-Affiliated Research Laboratory.

Subjects: Sprague Dawley Rats.

Interventions: Polyethylene glycol-20k.

Measurements and Main Results: Buccal microcirculation was measured at baseline, 1, 3, and 6 hours after return of spontaneous circulation using a side-stream dark-field imaging device. Myocardial function was measured by echocardiography at baseline and every hour postresuscitation for 6 hours. The animals were then returned to their cage and observed for an additional 72 hours. Neurologic Deficit Scores were recorded at 24, 48, and 72 hours after resuscitation. Postresuscitation ejection fraction, cardiac output, and myocardial performance index were significantly improved in animals treated with polyethylene glycol-20k ($p < 0.05$). Perfused buccal vessel density and microcirculatory flow index values were significantly higher at all time points in the polyethylene glycol-20k group compared with the control group. Postresuscitation cerebral function and survival rate were also significantly improved in animals that received polyethylene glycol-20k.

Conclusions: Administration of polyethylene glycol-20k following cardiopulmonary resuscitation improves postresuscitation myocardial and cerebral function, buccal microcirculation, and survival in a rat model of cardiopulmonary resuscitation. (*Crit Care Med* 2018; 46:e1190–e1195)

Key Words: cardioprotection; cerebral function; microcirculation; myocardial function; polyethylene glycol-20k; postresuscitation

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Sudden cardiac arrest (SCA) is a major public health concern in the United States with about 110.8 individuals per 100,000 affected every year (1). Current advanced cardiac life support therapies are only able to resuscitate less than 5% of victims despite substantial efforts to improve treatment of SCA (2). Postresuscitation myocardial and cerebral dysfunctions are responsible for most deaths that occur after resuscitation (3). Active protection of both myocardial and cerebral

function is therefore critical for improvement of postresuscitation outcomes.

Cell swelling induced by ischemia injury is one of the primary underlying mechanisms involved in ischemia-induced myocardial and cerebral dysfunction (4–6). Alleviation of cell swelling has been considered an effective strategy against ischemia-induced organ dysfunction (7–9). Cell impermeants have been widely used in modern organ preservation solutions, such as the University of Wisconsin cold storage solution, which have been shown to prevent cell swelling in cold-stored organs for transplantation and improve outcomes (10–13). Polyethylene glycol-20k (PEG-20k), a cell impermeant, is known to alleviate ischemia-induced cell swelling and interstitial edema due to its unique size and osmotic reflection coefficient (15). PEG-20k has been shown to significantly improve outcomes in a rat model of hemorrhagic shock by reducing ischemia-induced cell swelling, which dramatically improves capillary blood flow, oxygen exchange, and lactate clearance (14–17). In this study, we hypothesize that PEG-20k provides protective effects on neurologic and cardiac function and improves survival duration in a postresuscitation rat model due to potentially shared mechanisms of tissue injury involving metabolic cell swelling after ischemia (18–20).

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health.

Animal Preparation

Male Sprague Dawley rats, 6–8 months old weighing between 450*g* and 550*g*, were supplied by a single source breeder (Envigo, Frederick, MD). The animals were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg). Additional doses (10 mg/kg) were administered at hourly intervals or when required to maintain anesthesia. Depth of anesthesia was monitored by tail pinch. The trachea was orally intubated with a 14-gauge cannula mounted on a blunt needle (Abbocath-T; Abbott Hospital Products Division, North Chicago, IL) with a 145° angled tip. End-tidal CO_2 (Et_{CO_2}) was continuously monitored with a side-stream infrared CO_2 analyzer (Capstar-100 Carbon Dioxide Analyzer; CWE, Ardmore, PA). A conventional lead II electrocardiogram (ECG) was continuously monitored. For blood pressure measurements within the descending aorta and right atrium and drug administration, polyethylene catheters (PE-50; Becton Dickinson, Sparks, MD) were advanced into the descending aorta from the left femoral artery, the right atrium from the left external jugular vein, and the inferior vena cava from the right femoral vein, respectively. A thermocouple microprobe (IT-18; Physitemp Instruments, Clifton, NJ) was inserted into the descending aorta from the right femoral artery to detect blood temperature. A 3F catheter (Model C-PMS-301J; Cook Critical Care, Bloomington, IN) was advanced through the right external jugular vein into the right atrium. A precurved guidewire supplied with the catheter was then advanced through the catheter into the right ventricle to induce ventricular fibrillation (VF), and placement of the guidewire was confirmed by an endocardial electrocardiograph. All catheters were flushed intermittently with saline containing 2.5 IU/mL of crystalline bovine heparin. The core temperature (aortic blood temperature) was maintained at 37°C ± 0.5°C by a heating blanket.

Experimental Procedures

Rats were randomized into two groups: 1) control group (n = 10), 2) PEG-20k group (n = 10). Saline (2 mL) or PEG-20k (10% weight/volume, 2 mL) was administered at the start of cardiopulmonary resuscitation (CPR) by continuous IV infusion for 2 minutes with an infusion pump (GenieTouch; Kent Scientific, Torrington, CT). The investigators involved in CPR were blinded to group randomization. According to previous studies (14–17), 10% PEG-20k (given at 10% blood volume) has been proven to be optimal in a hemorrhagic shock model. However, too much loading volume during CPR is detrimental, due to decreased coronary perfusion pressure (CPP) resulting from increased pressure of the right atrium (21, 22). Our pilot study demonstrated that 2 mL is a sufficient dose to alleviate cell swelling without causing significant changes in hemodynamics.

Fifteen minutes prior to induction of VF, baseline measurements, and echocardiography were obtained. Mechanical ventilation was established at a tidal volume of 0.60 mL/100*g* of body weight, a frequency of 100 breaths/min, and F_{IO_2} of 0.21. Mechanical ventilation was discontinued after onset of VF. VF was then induced through a guide wire advanced from the right jugular vein into the right ventricle. A progressive increase in 60-Hz current to a maximum of 3.5 mA was then delivered to the right ventricular endocardium. The current flow was continued for 3 minutes to prevent spontaneous defibrillation. After 6 minutes of untreated VF, precordial chest compressions, together with mechanical ventilation (tidal volume 0.60 mL/100*g* body weight, frequency 100 breaths/min, F_{IO_2} 1.0), were initiated using a pneumatically driven mechanical chest compressor. Precordial chest compressions were maintained at a rate of 200/min and synchronized to provide a compression/ventilation ratio of 2:1 with equal compression-relaxation for a duration of 8 minutes. Defibrillation was attempted with up to three 4-J counter shocks after 8 minutes of CPR. Return of spontaneous circulation (ROSC) was defined as the return of supraventricular rhythm with a mean aortic pressure above 50 mm Hg for 5 minutes. If ROSC was not achieved after the first defibrillation attempt, a 30-second interval of CPR was performed prior to the next defibrillation attempt (up to three attempts). After ROSC, an F_{IO_2} of 1.0 was continued for 1 hour, adjusted to 0.5 for the second hour, and 0.21 thereafter.

Measurements

ECG, aortic and right atrial pressures, Et_{CO_2}, and blood temperature values were continuously recorded on a personal computer–based data acquisition system supported by WINDAQ software (DATAQ, Akron, OH). CPP was calculated

as the difference in time-coincident diastolic aortic and right atrial pressures that were displayed in real time.

Myocardial function, including cardiac output (CO), ejection fraction (EF), and myocardial performance index (MPI), was measured at baseline and hourly after ROSC for a total of 6 hours by echocardiography (HD11XE; Philips Medical Systems, Eindhoven, the Netherlands) with a 12.5 Hz transducer. CO and EF were used to estimate myocardial contractility; MPI was used to estimate left ventricular diastolic function. All measurements were reviewed and confirmed separately by two investigators blinded to the groups.

Buccal microcirculation was measured at baseline, 1, 3, and 6 hours after ROSC using a side-stream dark-field imaging device (MicroScan; MicroVision Medical, Amsterdam, the Netherlands) that had a 5× imaging objective, resulting in an on-screen magnification of 276×. Three discrete fields for each were captured with the intention to minimize motion artifacts. Microvascular images were recorded on a DVD with a DVD recorder (DMR-EZ47V; Panasonic AVC Networks, Dalian, China). Microcirculatory flow index (MFI) was measured using the method of Spronk et al (23). The image was divided into four quadrants, and the predominant flow type (absent = 0, intermittent = 1, sluggish = 2, normal = 3) was assessed in the small vessels of each quadrant, which were less than 20 μm in diameter. The MFI score represented the average values of the four quadrants. Perfused vessel density (PVD) was quantitated based on the method of De Backer et al (24). Vessel density was calculated as the number of vessels crossing the catheters divided by the total length of the catheters. All recordings were analyzed by three independent observers blinded to the groups.

Neurologic Deficit Score (NDS), which ranged from 0 (no observed neurologic deficit) to 500 (death or brain death), was used to evaluate neurologic function (25). The NDSs were examined and confirmed by two investigators blinded to treatment at 24, 48, and 72 hours after resuscitation.

Statistical Analysis

All data were presented as mean ± SD. Two-way analysis of variance was performed to determine differences between groups in all time points. Log-rank (Mantel-Cox) test was used for survival analysis. A value of p less than 0.05 was regarded as significant.

RESULTS

A total of 25 rats were used in this study. Twenty rats were successfully resuscitated and included for analysis. Five rats were excluded, which consisted of four rats that were not resuscitated and one rat that was excluded due to instrumentation or technical failure during animal preparation. There were no significant differences in hemodynamics, blood temperature, body weight, Et_{CO_2}, myocardial function (EF, CO, and MPI), and buccal microcirculation (MFI and PVD) at baseline between the two groups. There were no significant differences in CPP and Et_{CO_2} during CPR between the two groups. No significant difference was observed in temperature throughout the experiment.

After resuscitation, myocardial function, as indicated by EF, CO, and MPI, was significantly impaired in both groups when compared with baseline values (**Fig. 1**). Significant improvement in myocardial function was observed in the PEG-20k group at all time points when compared with the control group (Fig. 1). Buccal microcirculation was reduced significantly after successful resuscitation in both groups compared with baseline values (**Fig. 2**). PVD and MFI values were significantly higher at all time points in the PEG-20k group compared with the control group (Fig. 2).

NDS was used to assess neurologic function after resuscitation. As shown in **Figure 3**, lower NDS values were observed at 24, 48, and 72 hours after resuscitation in the PEG-20k group compared with the control group. As shown in **Figure 4**, overall survival rates were significantly higher in the PEG-20k group compared with the control group. Seven rats survived through the entire 72-hour period in the PEG-20k group, compared with the one rat that survived through the 72-hour period in the control group. Most rats died within the first 24 hours in the control group.

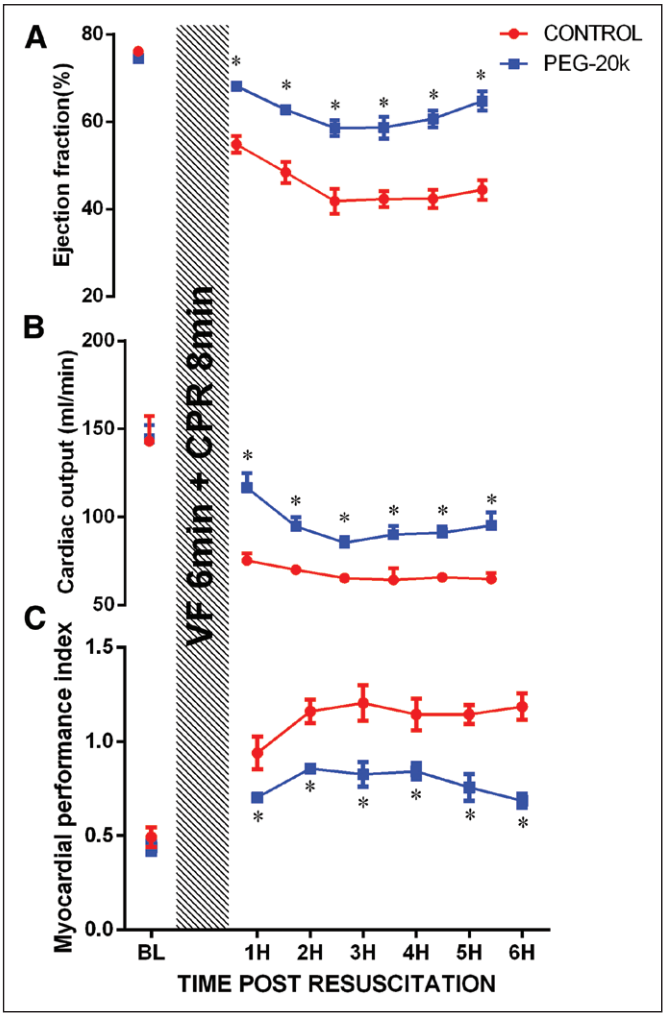

Figure 1. Polyethylene glycol-20k (PEG-20k) improves postresuscitation myocardial function. **A**, Ejection fraction, **B**, cardiac output, and **C**, myocardial performance index. *$p < 0.05$ versus Control Group-CA with administration of saline (2 mL) at the start of cardiopulmonary resuscitation (CPR) by continuous IV infusion for 2 minutes (CONTROL) group. BL = baseline, H = hour, VF = ventricular fibrillation.

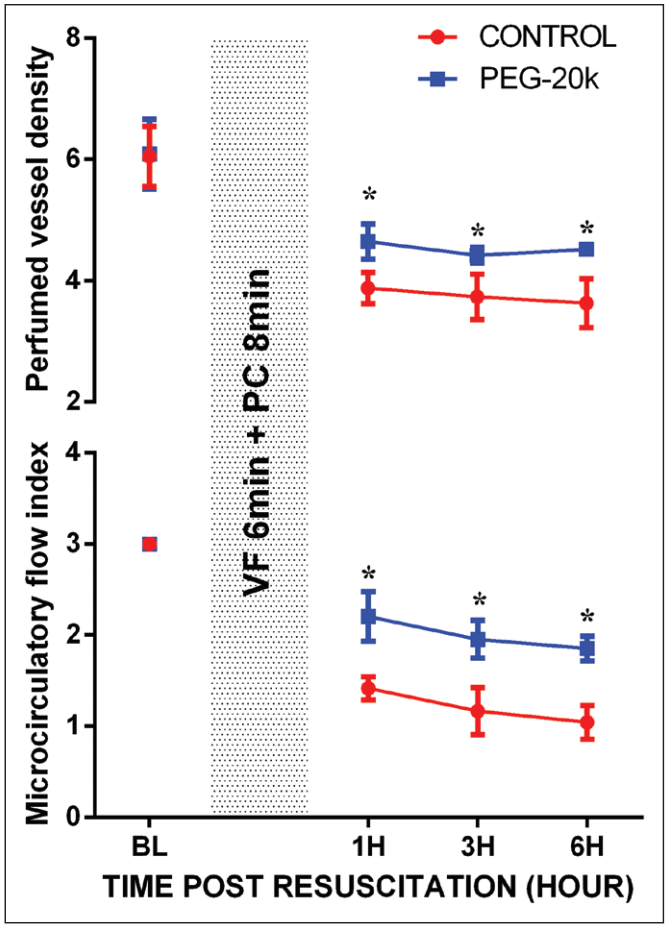

Figure 2. Polyethylene glycol-20k (PEG-20k) improves buccal microcirculation. **A**, Perfused vessel density, and **B**, microcirculatory flow index. *$p < 0.05$ versus Control Group-CA with administration of saline (2 mL) at the start of cardiopulmonary resuscitation by continuous IV infusion for 2 minutes (CONTROL) group. BL = baseline, H = hour, PC= precordial compression, VF = ventricular fibrillation.

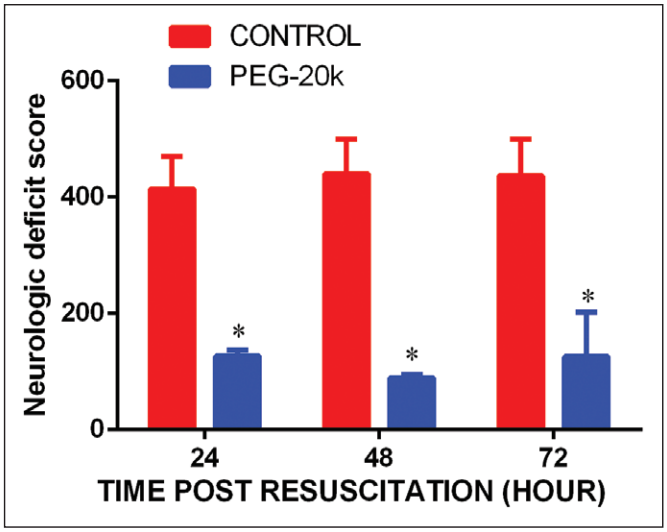

Figure 3. Polyethylene glycol-20k (PEG-20k) improves postresuscitation cerebral function. *$p < 0.05$ versus Control Group-CA with administration of saline (2 mL) at the start of cardiopulmonary resuscitation (CPR) by continuous IV infusion for 2 minutes (CONTROL) group.

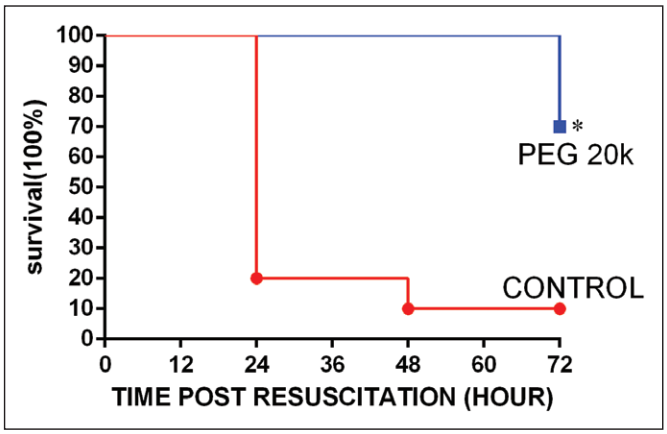

Figure 4. Polyethylene glycol-20k (PEG-20k) improves survival duration. *$p < 0.05$ versus Control Group-CA with administration of saline (2 mL) at the start of cardiopulmonary resuscitation (CPR) by continuous IV infusion for 2 minutes (CONTROL) group. BL = baseline.

DISCUSSION

The present study demonstrates significantly improved postresuscitation myocardial and cerebral function, buccal microcirculation, and duration of survival in animals treated with PEG-20k during CPR. These outcomes likely occurred due to alleviation of ischemia-induced cellular edema by PEG-20k.

Substantial myocardial and cerebral dysfunctions after successful resuscitation from cardiac arrest are associated with high postresuscitation mortality rates. This has been documented in clinical studies (26, 27). Likewise, postresuscitation myocardial and cerebral functions were significantly impaired in the present study, leading to shortened survival duration in the control group.

Energy failure during global ischemia is one of the primary mechanisms underlying postresuscitation organ dysfunction. Inadequate oxygen transport resulting in inefficient adenosine triphosphate (ATP) synthesis during ischemia leads to cellular energy depletion and failure of the ATP-dependent Na/K ATPase pump is instrumental in ischemia reperfusion injury. The subsequent influx of sodium followed by entry of extracellular fluid into the cells through osmosis results in cellular edema (6). Eventual hydropic degeneration leads to the disruption of cell and organelle membranes, culminating in disturbances in cellular homeostasis and cell death (28). Cell swelling within the parenchyma causes compression of local capillaries, resulting in a vicious cycle with further reductions in capillary flow and oxygen delivery. Cell and tissue swelling during resuscitation also contribute to the "no reflow phenomenon," exacerbating the ischemic cycle (29–32).

The role of ischemia-induced cellular edema in organ dysfunction is recognized in organ preservation injury (12, 30–34). Cell impermeants are widely used in organ preservation solutions to reduce cell swelling and confer protective effects on organ function (38, 39). Polyethylene glycol (PEG) is a linear polymer composed of repeating ethylene glycol units. Its molecular weight, dependent on the number of ethylene glycol units, is associated with permeability (38, 39). PEG is nontoxic to animals once above 400 daltons and is impermeable to cells above 500 daltons (39–41). Polymers above 80,000 daltons are

confined to the capillary space where they act as colloids (42). Polymers between 20,000 and 35,000 daltons have variable permeability within the capillary space, giving them distinct oncotic strengths. At 20,000 daltons, PEG-20k is a true hybrid molecule, acting as both a cell impermeant and an oncotic agent due to its unique size and molecular radius. With a capillary oncotic reflection coefficient of approximately 0.65, PEG-20k remains primarily within the capillary where it exerts its oncotic actions. It is also able to cross into the interstitial space where it acts as a cell impermeant (14, 15). Therefore, PEG-20k can drive water from the cells and interstitial space into the capillary space more efficiently than either pure colloids alone or pure impermeants alone. Establishing this double osmotic gradient in the microcirculation alleviates cellular, tissue, and interstitial edema during global ischemia, decompresses the microcirculation, and dramatically improves capillary oxygen transfer at reperfusion (17). In several hemorrhagic shock models, PEG-20k has been shown to prevent ischemia-induced cell swelling, resulting in dramatically improved outcomes (14–16). If this also occurs in the ischemic myocardium during CPR, then this may be a plausible mechanism of action that explains the salutary effects on outcomes in our model. In fact, the significantly improved buccal capillary perfusion after PEG-20k supports this hypothesis.

The brain is especially sensitive to cellular edema, as it is confined to an inflexible space (6). Ischemia-induced cell swelling within the brain after resuscitation elevates intracranial pressure, leading to diminished cerebral blood flow with subsequent loss of brain function. Alleviation of cerebral edema is associated with improved postresuscitation outcomes after cardiac arrest (7, 43). Similarly, ischemia-induced swelling of cardiac myocytes results in myocardial edema and subsequent cardiac dysfunction (9, 44). PEG-20k has been shown to alleviate cell swelling in organ preservation and hemorrhagic shock models. The present study further revealed its significant protective effects on myocardial and cerebral function and the microcirculation, ultimately leading to improved survival duration in a rat model of cardiac arrest and resuscitation. Under normal physiologic conditions, PEG-20k cannot cross the blood brain barrier; therefore, the improved cerebral function seen in the present study may be due to an impaired blood brain barrier during cardiac arrest and resuscitation, as previously reported (45–48).

This is the first study to evaluate the effects of PEG-20k on postresuscitation myocardial and cerebral function and duration of survival in a rat model of cardiac arrest and resuscitation. The present study demonstrated significantly improved postresuscitation outcomes in the treatment group, with improved myocardial and cerebral function and microcirculation. Active protection of the heart and brain by PEG-20k during cardiopulmonary resuscitation is due to the alleviation of ischemia-induced cell swelling, allowing for decompression of microcirculation and restoration of microcirculatory flow. Water drawn into the capillary space through its oncotic actions improves local tissue perfusion and retards the accumulation of oxygen debt and subsequent energy depletion. Cell impermeants such as PEG-20k provide an effective potential treatment for early and rapid protection of vital organs during cardiopulmonary resuscitation. PEG-20k may be a viable and innovative therapeutic option to decrease postresuscitation mortality rates after cardiac arrest.

An alternative hypothesis to explain the salutary effects of PEG-20k solutions in myocardial protection after CPR is through protection of the endothelial glycocalyx. Ischemia and resuscitation, especially with crystalloids, can cause erosion of the endothelial glycocalyx with subsequent activation of inflammatory responses at reperfusion. Since PEG polymers are able to bind to cell surface molecules, it is conceivable that they may bind to and protect the glycocalyx proteoglycan constituents or even serve to rebuild it after it erodes.

There are some limitations in this study. First, only rats without underlying disease were included, which is not consistent with clinical conditions. Second, PEG-20k was administered at the very beginning of CPR, which may not be applicable to human victims. Third, toxicity analysis needs to be investigated before it is proposed for future human clinical use. Fourth, histologic analysis of correlates to demonstrate attenuation of brain injury was not performed and should be investigated in future studies.

CONCLUSIONS

Administration of PEG-20k following CPR improves postresuscitation myocardial and cerebral function, buccal microcirculation, and survival in a rat model of CPR. Further study will be needed to determine the optimal, dose, timing, and mechanism of action of PEG-20k's apparent protective effects after resuscitation.

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PLOS ONE

RESEARCH ARTICLE

Effects of a novel low volume resuscitation solutions on coagulation and platelet function

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Abstract

Background

Novel crystalloid solutions containing polyethylene glycol polymers (PEG-20k) produce dramatic resuscitation effects but dose-dependently produce a hypocoagulative state. The objective of this study was to examine possible mechanisms of this effect. Based on previous thromboelastography data, we hypothesize the effect is largely due to platelet interactions with the polymers.

Methods

Whole citrated blood from healthy volunteers was diluted ex-vivo 10% with crystalloids and tested for coagulation and platelet function. The specific tests included prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen and von Willebrand factor (vWf) activity, thrombin generation, thromboelastography with and without platelet mapping, platelet flow cytometry, and erythrocyte sedimentation rate.

Findings

Fibrinogen and vWF activities, PT, and aPTT were not affected by PEG-20k dilutions. Thrombin activity was mildly suppressed with PEG-20k (TTP- 20%). Platelet mapping demonstrated significantly greater % inhibition of both ADP and arachidonic acid-induced platelet aggregation with PEG-20k, but direct ADP-activated gpIIa/IIIb (PAC1) and P-selectin (CD62P) binding site expression was not altered. Mild dose-dependent suppression of TEG-MA was seen with PEG-20k using platelet poor plasma. Erythrocyte Sedimentation Rates (ESR) were dramatically accelerated after dilution with 10% PEG-20k, which was competitively blocked by smaller PEG polymers, suggesting nonspecific PEG-20k cell binding effects.

decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: vWF, von Willebrand Factor; LVR, Low Volume Resuscitation; PEG-20k, Polyethylene Glycol 20,000 Da; NS, 0.9% NaCl; LR, Lactated Ringers; HES, Hetastarch; TEG, Thromboelastography; TICS, Trauma-Induced Capillary Leak Syndrome; PPP, Platelet Poor Plasma; ISS, Injury Severity Scor.

Conclusions

PEG-20k creates a mild hypocoagulative state in whole blood at concentrations $\geq$10%, which may be due to platelet-PEG interactions at the IIb/IIIa interface with lesser effects on fibrin polymerization. This interaction may cause a functional thrombasthenia induced by nonspecific platelet surface passivation by the PEG polymer.

Introduction

Trauma is the number one cause of death for people under 44 years of age in the US and the third leading cause of death overall for all age groups. Trauma accounts for about 30% of all life-years lost in the US, compared to cancer (16%), heart disease (12%), and HIV (2%) [1]. For all traumatic injuries, hemorrhagic shock is responsible for over 35% of pre-hospital deaths and over 40% of all deaths within the first 24 hours. This is second only to deaths induced by severe CNS injury [2]. Hemorrhagic hypotension exposes the patient to immediate complications of life-threatening infections, coagulopathies, and multiple organ failure [3, 4].

Crystalloid-based intravenous (IV) solutions are available for pre-hospital use because they can be safely transported and stored but they are generally limited in their effectiveness. Only a fraction of infused crystalloid volume stays in the intravascular space and the use of low volume crystalloids has minimal effects on pressure and perfusion [5, 6]. The movement of crystalloid fluid from capillary to interstitium is compounded by the increase in capillary permeability from trauma-related inflammation and trauma-induced capillary leak syndrome (TICS) [7]. Furthermore, crystalloid resuscitation exacerbates TICS, acidosis, hypothermia, and coagulopathy [7, 8]. Other resuscitation solutions such as hypertonic saline or starch have had disappointing results [9, 10] including concerns and risks associated with their use [8, 11]. There remains a need for a better crystalloid fluid that can be given at a low volume to resuscitate patients in severe hemorrhagic shock awaiting definitive treatment, especially for the pre-hospital setting.

Recently, polyethylene glycol (PEG) polymers of specific molecular weight ranges have been used in crystalloid solutions to act as highly effective low-volume resuscitation (LVR) solutions [6, 12–14]. These polymers non-energetically move isotonic fluid from intracellular and interstitial spaces into the capillary space by simple osmotic actions in response to metabolic cell swelling that occurs in shocked and ischemic tissues. As water flow moves from the interstitial spaces to the capillaries, the capillary exchange in the tissues dramatically improves under very low volume conditions because the microcirculation is decompressed while the capillary spaces are re-loaded with volume and pressure for driving flow [14]. This causes rapid clearance of lactate, increased blood pressure, and tolerance to the low volume state [12]. While these polymers work several-fold better than hydroxyethyl starch based polymers [6, 13, 14], implying different mechanisms of action, interference with blood clotting and coagulation may be shared by both types of polymers. For example, the I.V. starch-based crystalloid solutions Hextend and Hespan are complicated by both renal toxicity and coagulopathies [15], which in trauma settings are a concern.

In a set of experiments recently published [16], we described detailed thromboelastography (TEG) evidence of a mild hypocoagulative state induced by 10% dilutions of blood samples from healthy volunteers and from blood samples from trauma patients with 10% PEG-20,000 Da (PEG-20k) solutions. The TEG-based data suggested PEG-20k had effects on not only final clot strength (maximal amplitude, MA), but also on the clot propagation parameters *k* and α-

angle, which are measurements influenced by fibrinogen cross-linking. The PEG-20k effects on TEG parameters were significantly different, relative to those of normal saline and hetastarch, and appeared in a dose-dependent fashion.

Therefore, the aim of this study was to characterize the mechanism of the dose-dependent hypocoagulopathy findings in the TEG parameters observed with PEG-20k solutions. To that end, we systematically studied a battery of coagulation and platelet function parameters in blood samples obtained from healthy volunteers diluted 10% with PEG-20k solution. From our previous TEG analysis, we hypothesize that the hypocoagulable state induced by PEG-20k solutions on whole clotting blood is related mainly to interferences of the polymer with platelet function.

Methods

Volunteer blood

This study was done under the approval of the VCU Institutional Review Board (approval number HM20002817). Each blood donor for this study provided written consent. Whole blood (15-ml) was drawn into citrated vacutainer collection tubes from 12 healthy consented volunteers (18–50 years of age) of both sexes that were free of all medications and tobacco. All volunteers donated blood under a VCU approved IRB protocol. The blood was diluted 10% in the lab with solutions of 10%, 7.5%, and 5% PEG-20k in saline (0.9% NaCl), 6% Hextend, or a 0.9% NaCl solution that served always as a paired dilutional control for all test solutions. Immediately after the dilutions, the samples were analyzed for coagulation parameters. One mL of citrated whole blood aliquots was used for TEG analysis using kaolin as activator, and the remaining citrated whole blood was centrifuged at 180 x g for 10 minutes to obtain platelet rich plasma (PRP). Platelet poor plasma (PPP) was obtained by double centrifugation of the remaining plasma at 2000 x g for 10 min at room temperature. PRP was then diluted with autologous PPP to yield a final platelet count of 150 $x10^9$/L for platelet-dependent thrombin generation assays. The remaining PPP was used for the analysis of platelet-independent thrombin generation, PT, aPTT, fibrinogen, and vWF concentrations. Platelet counts were performed with an automated cell counter (ABX Micros 60, Horiba Medical, Irvine, CA, USA). The time between blood draw and analysis was less than two hours. Normal values have been previously described [17–20].

Fibrinogen, PT, aPTT, vWF

Fibrinogen, PT, aPTT, and von Willebrand factor antigen (vWF) function were measured in plasma using standard assays (STA fibrinogen clotting activity assay, PT-Neoplastin CI, PTT-Automate, PTT CK Prest, and Liatest vWF assays, respectively) on the STA Compact analyzer (Diagnostica Stago, Parsippany, NJ, USA) according to manufacturer's instructions.

Thrombin generation assay

The kinetics of thrombin generation was assessed in PRP and in PPP according to methods previously described by Hemker, et al [19]. Briefly, 20 μl of trigger reagent (1pM Tissue Factor), and 80 μl PPP were manually pipetted in triplicate into 96-well microtiter plates (Immulon 2HB plate; Diagnostica Stago, Parsippany, NJ, USA). The plate was placed in the fluorometer for a 10 minute 37˚C incubation (Fluoroskan AscentTM; Thermolab Systems OY, Helsinki, Finland). The device was equipped with a 390/460 filter set. Twenty μl of starting reagent containing the fluorogenic substrate Z-GGR-AMC (2.5 mM) and CaCl2 (100 mM) were automatically dispensed into each well immediately before measurement initiation.

Thrombin generation curves were calculated using the calibrated automated thrombogram (Thrombinoscope BV, Masstricht, The Netherlands) software version: V5.0.0.742. The thrombogram parameters (lag time, peak thrombin concentration, and endogenous thrombin potential (ETP), which reflected the maximum amount of thrombin that a sample could potentially generate) were reported.

TEG and platelet mapping

Thromboelastography with platelet mapping was determined using a TEG 5000 (Haemonetics Corp., Braintree, Mass) using the intrinsic pathway activator kaolin (Haemonetics Corp.) and recalcification to 10 mmol/L final calcium concentration. The TEG 5000 reported time to onset of clot formation (R), which positively correlates with thrombin generation; the time to reach a predetermined level of clot stiffness (K) and the clotting angle (α-angle), which correlates with fibrin polymerization; the maximal amplitude (MA) or stiffness, representing clot strength. Platelet mapping was done using the TEG-5000 instrument and a platelet mapping kit (Haemonetics Corp.) that tests the platelet component to clot formation (MA) on TEG [21]. Briefly, heparinized blood treated with reptilase and activated factor XIII was used to form thrombin independent cross-linked fibrin. The platelet specific component for the adenosine diphosphate (ADP) and thromboxane receptor pathways were determined by activation with ADP and arachidonic acid (AA), respectively, on the heparinized blood samples. All assays were performed according to manufacturer guidelines.

TEG on platelet poor plasma (PPP)

To test the effects of PEG-20k on just the fibrin component of clot formation, TEG was performed on platelet poor plasma. Citrated whole blood was drawn from six healthy volunteers as before and the plasma was obtained by centrifugation at 2500 x g for 10 minutes. The PPP was diluted 10% with solutions of PEG-20k at 0%, 5%, 10%, and 15%. Standard TEG using kaolin activation was performed as before on each sample immediately after PEG-20k dilution and compared to the 10% dilutional volume control (0% PEG-20k) using saline.

Platelet flow cytometry

Platelet activation was also quantified using flow cytometric analysis. Briefly, flow cytometry was performed on a BD Biosciences device (BD Biosciences Accuri™ C6 Flow Cytometer, San Jose, CA, USA) using citrated whole blood according to current standards from the European Working Group on Cell Analysis [22]. CD41a conjugated with PE-Cy5 (Mouse Anti-Human, BD Pharmingen, Franklin Lakes, NJ, USA), PAC-1 conjugated with FITC (BD Biosciences), and CD62p conjugated with PE (Mouse Anti-Human, BD Pharmingen) were used to identify platelets and to identify their activation status. Corresponding isotypic-matched monoclonal antibodies PE-Mouse IgG1-K isotype, FITC-Mouse IgM-K isotype and PE-Cy5-Mouse IgG1-K isotype (BD Pharmingen) were used as negative controls. A portion of the whole blood specimens was treated with 0.005 mL of ADP (3 μM final) for platelet activation. Samples were analyzed under the following conditions: Fluidics: medium; Forward scatter threshold: 30,000; and 20,000 events were collected in a preset platelet gate using standard methods including CD41a as a global platelet marker. Results are expressed in mean fluorescence intensity units for CD41 and in percentages for other markers of activation. Flow cytometry measured platelet activation via the glycoprotein P-selectin, because it rapidly translocates to the platelet surface on stimulation. The P-selectin content on the platelet surface was detected with the CD62-P mAb. Also measured was the glycoprotein IIb/IIIa surface integrin transition to

its high-affinity state by using the mAb against high-affinity glycoprotein IIb/IIIa platelet surface integrin (PAC-1) conjugated with fluorescein isothiocyanate (BD Biosciences).

ESR

The erythrocyte sedimentation rate was measured in diluted citrated whole blood using the Sediplast Westergren ESR system tests (Polymedco, Inc., Cortiandt Manor, NY). About 1 ml of whole blood was drawn up into a 10 cm Westergren ESR tube, which was held in the vertical position for 75 minutes. The rate of red blood cell sedimentation was measured as the migration (in mm) of the red cell column down the tube under the force of gravity. Blood samples diluted with 10% volume of saline (volume control) were compared to 10% dilution with PEG-20k solutions and PEG-20k solutions with other test compounds.

Statistical data analysis

All statistical analysis was performed using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA). Data groups were analyzed for outliers using the nonlinear regression ROUT method with Q = 1%, the maximum desired false discovery rate. Normality of Gaussian distribution was then assessed using the D'Agnostino-Pearson ombinus K2 method. Most data were then analyzed by the non-parametric ANOVA Kruskal-Wallis test with the Mann Whitney U test for multiple comparisons of means. The data are presented as either mean (standard deviation) or as median with interquartile ranges. The ESR competitive inhibitor data were analyzed by nonlinear regression analysis. A p-value < 0.05 was considered statistically significant.

Results

The plasma concentrations of fibrinogen and von Willebrand factor (vWF) activity obtained from healthy volunteers that had been diluted 10% with either PEG-20k (10% w:v) or a saline dilution control are shown **Fig 1.** There was no difference in fibrinogen concentrations due to dilution with PEG-20k (Panel A). However, there was a small but statistically significant decrease in vWF activity observed for the PEG-20k (Panel B) samples but the levels in this group were still within the normal range.

Fig 2 shows the PT (Panel A) and aPTT (Panel B) when plasma was diluted by 10% with either PEG-20k or saline. This is the theoretical dilution that occurs when the solutions are administered to shocked patients. These data illustrate that PEG-20k has no effect on neither PT, nor aPTT when the activator reagents included a combination of kaolin and rabbit brain phospholipids. However, when the activator for this test contained micronized silica instead of kaolin and rabbit brain phospholipids, there was a very significant prolongation in the aPTT in the PEG-20k diluted plasma samples, relative to the saline controls, suggesting that a silica-PEG-20k interaction exists that interferes with initiation of the intrinsic pathways cascade. So while PEG-20k did not have any significant effects on coagulation, it dramatically prolongs the aPTT times when micronized silica is used as an activator. This interaction has recently been observed in PEG-conjugated compounds including PEGylated factor replacement products used for patients with hemophilia [23–25]. This technical complication should be avoided when testing PEG-20k diluted blood in clinical laboratories.

Fig 3 shows thrombin generation in PRP and PPP when the samples were diluted with either the PEG-20k or saline control. The presence of PEG-20k in the sample showed no effect on all CAT parameters except a slight but significantly prolonged thrombin generation lag time, but only in the PRP samples.

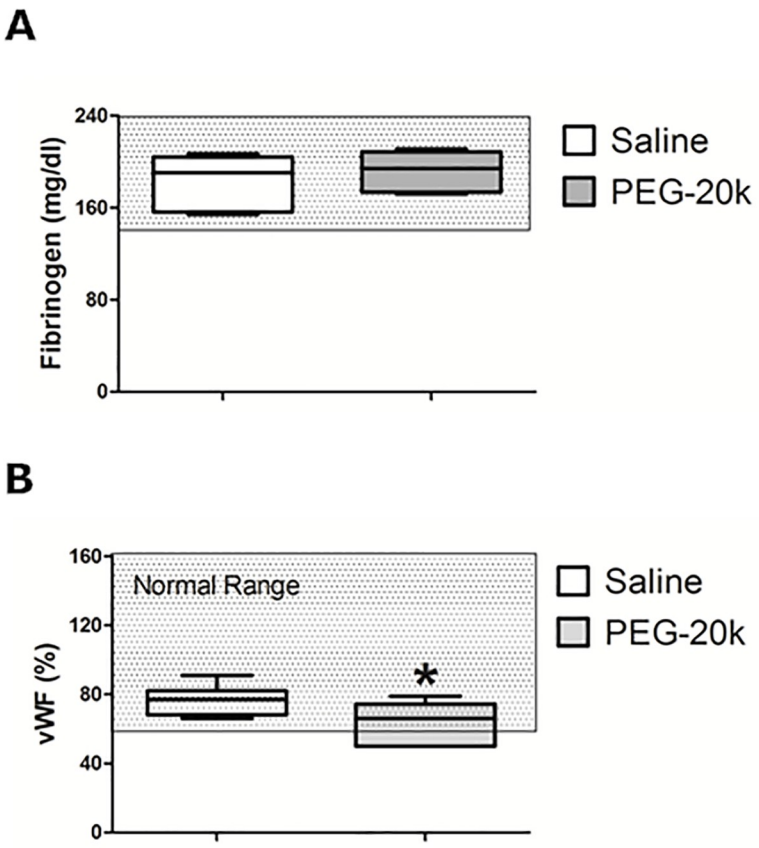

Fig 1. Plasma fibrinogen (panel A) and von Willebrand factor (panel B) concentrations in whole blood from healthy volunteers diluted with either saline (1 to 9 dilution) or 10% PEG-20k solution (1 to 9). The bar inside of the box is the median value of the sample, the lower and upper borders of the box represent the boundaries between the 1st and 2nd quartile and the 3rd and 4th quartile, respectively, and the ends of the whiskers indicate the population extreme values (low and high). Each group represents 6 individual values performed in triplicate. The shaded box is the normal range of values. $P<0.05$, relative to the saline dilutional control group [35].

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The results of TEG platelet mapping, showing the platelet contribution to clot formation under platelet stimulation with either ADP or arachidonic acid (AA), is shown in Fig 4. Dilution with 10% PEG-20k caused a significant decrease in the ADP and AA-induced aggregation response, relative to the saline control. This is expressed as the inhibition (%) of the maximal response observed in the absence of PEG-20k or the saline vehicle.

The flow cytometry data are presented in Fig 5. ADP activation of platelets in PRP induces a rapid expression of glycoprotein IIb /IIIA complexes and P-Selectin that are detected by specific binding of antibodies to PAC1 and anti-CD62P, respectively. While there were significant increases in both PAC1 (88.5%) and CD62p (59.7%) antibody binding to ADP-activated platelets, compared to the non-activated state with saline dilution, the effect was not different when PEG-20k was used as the diluent (87.4% increase for PAC1 and 62.5% increase for CD62p).

The effects of PEG-20k on just the chemical phase of blood coagulation is shown in Fig 6. Following removal of the platelet components by centrifugation, the platelet poor plasma component was assayed in a standard TEG experiment using kaolin activation. The MA and Angle components of TEG are reported for varying doses of PEG-20k from 0–15 mg/ml with a constant volume dilution of 10% for all tests. The absolute baseline clot and kinetics are smaller compared to values obtained from volunteer whole blood [16]. The clot MA and angle were

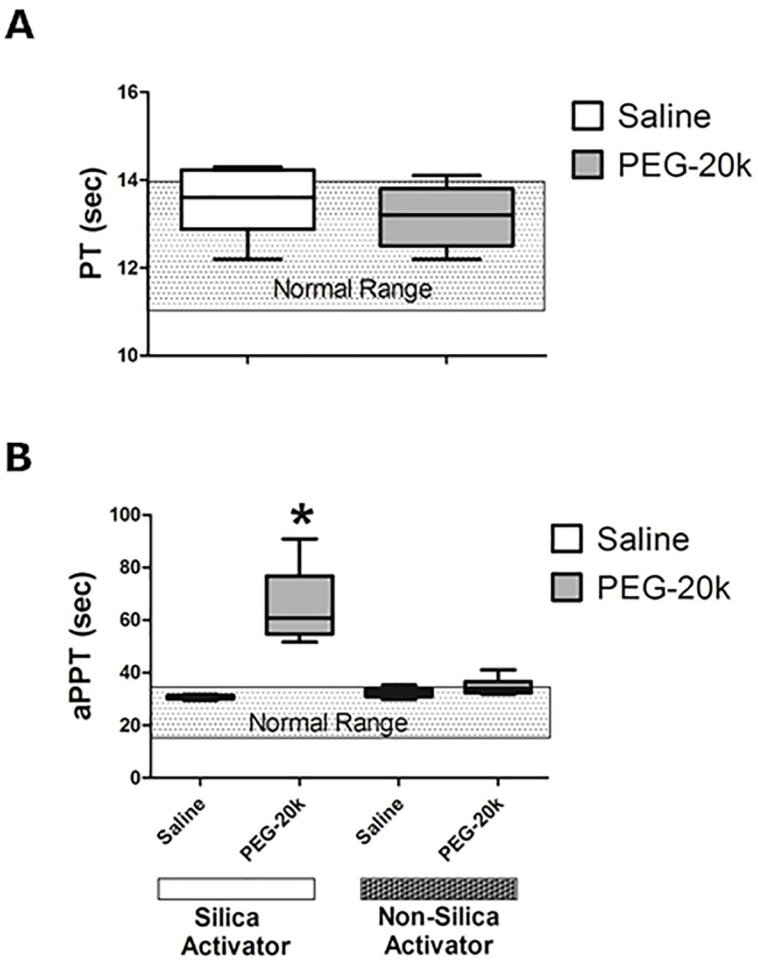

Fig 2. The plasma PT (panel A) and aPTT times (panel B) measured in blood from healthy volunteers diluted with either saline (1 to 9) or 10% PEG-20k solution (1 to 9). The bar inside of the box is the median value of the sample, the lower and upper borders of the box represent the boundaries between the 1st and 2nd quartile and the 3rd and 4th quartile, respectively, and the ends of the whiskers indicate the population extreme values (low and high). Each group represents 6 individual values. The shaded box is the normal range of values. $P<0.05$, relative to the corresponding saline dilutional control group. Panel B also shows the effects of micronized silica activator on aPTT compared to activators using kaolin.

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not significantly different from the dilutional control for 5 mg/ml PEG-20k but were different at the higher concentrations ($P<0.05$).

Finally, in an attempt to understand the cell binding effects of PEG-20k in blood, we used the erythrocyte sedimentation rate (ESR) as a model for what may be happening in the platelet fraction with PEG-20k (**Fig 7**). The ESR was significantly and dose-dependently increased with 7.5% and 10% PEG-20k solutions diluted 10% with whole blood (Panel A). Addition of 10% PEG-20k (weight to volume) induced a 150 fold increase in the rate of erythrocyte sedimentation, compared to the saline control at the same 10% volume dilution. In another study, the ESR sedimentation effect of 10% PEG-20k could be competitively inhibited by the addition of shorter chain PEG polymers of 1k, 4k, and 8k (8k shown in panel B).

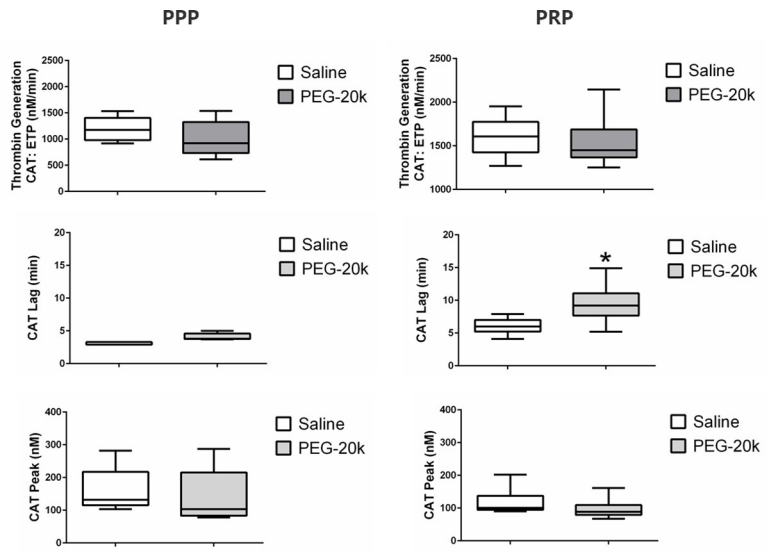

Fig 3. Plasma thrombin generation in PPP and PRP from healthy volunteers diluted with either saline (1 to 9) or 10% PEG-20k solution (1 to 9). CAT data shown include the ETF parameter, which is the area under the thrombin curve, the peak height of the thrombin curve, and the lag time from the time of activation until the start of thrombin generation. The bar inside of the box is the median value of the sample, the lower and upper borders of the box represent the boundaries between the 1st and 2nd quartile and the 3rd and 4th quartile, respectively, and the ends of the whiskers indicate the population extreme values (low and high). Each group represents 6 individual values. * $P<0.05$, relative to the saline dilutional control group.

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Discussion

PEG-20k, a new LVR crystalloid solution has recently been developed that induces profound tolerance to the low volume state when compared to other commonly used solutions. In preliminary testing using thromboelastography [16] it was determined that these solutions, which contain 10% PEG-20k, produced a dose-dependent hypocoagulative state, namely a significant decrease in MA and decreases in α-angle and k. Since MA represents clot firmness associated

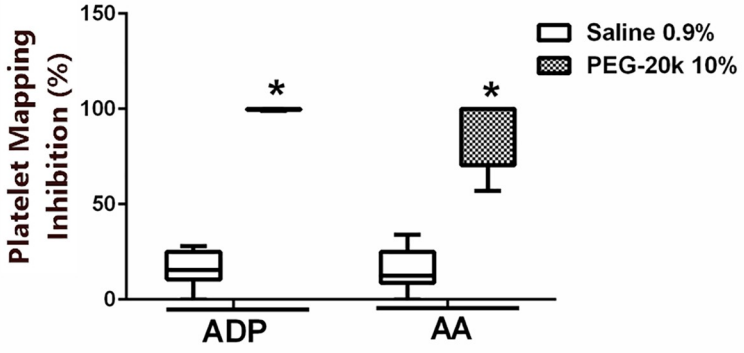

Fig 4. TEG platelet mapping studies conducted with whole blood obtained from volunteers diluted with either saline (1 to 9) or 10% PEG-20k solution (1 to 9). The figures shows the inhibition of activation of platelet clot formation in response to either ADP or arachidonic acid. The bar inside of the box is the median value of the sample, the lower and upper borders of the box represent the boundaries between the 1st and 2nd quartile and the 3rd and 4th quartile, respectively, and the ends of the whiskers indicate the population extreme values (low and high). Each group represents 6 individual values. $P<0.05$, relative to the saline dilutional control group.

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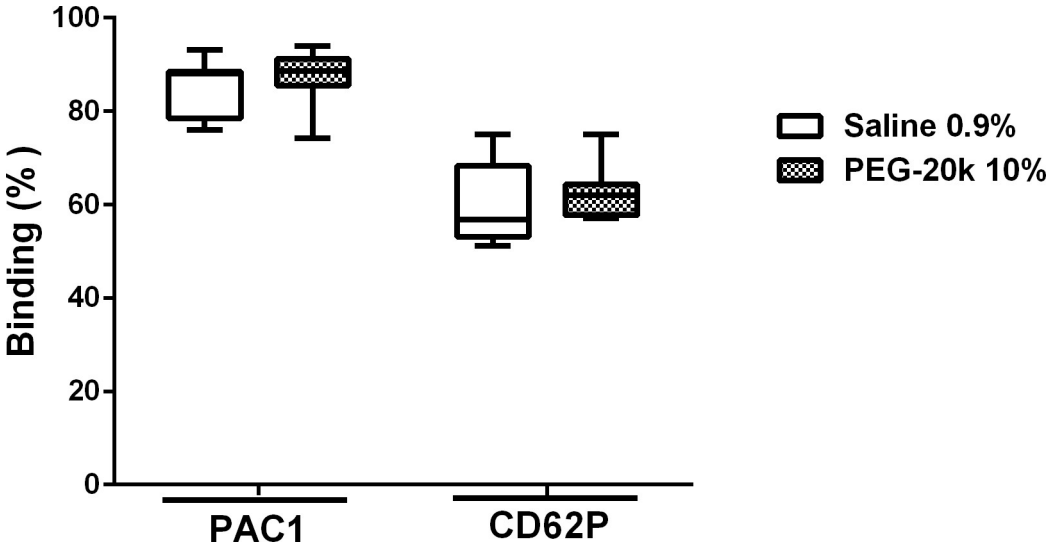

Fig 5. ADP-induced activation of expression of PAC1 (IIB/IIIA receptor complex) and CD62P (P-selectin) receptors on platelets in blood obtained from healthy volunteers diluted with either saline (1 to 9) or 10% PEG-20k solution (1 to 9). The bar inside of the box is the median value of the sample, the lower and upper borders of the box represent the boundaries between the 1st and 2nd quartile and the 3rd and 4th quartile, respectively, and the ends of the whiskers indicate the population extreme values (low and high). Each group represents 6 individual values.

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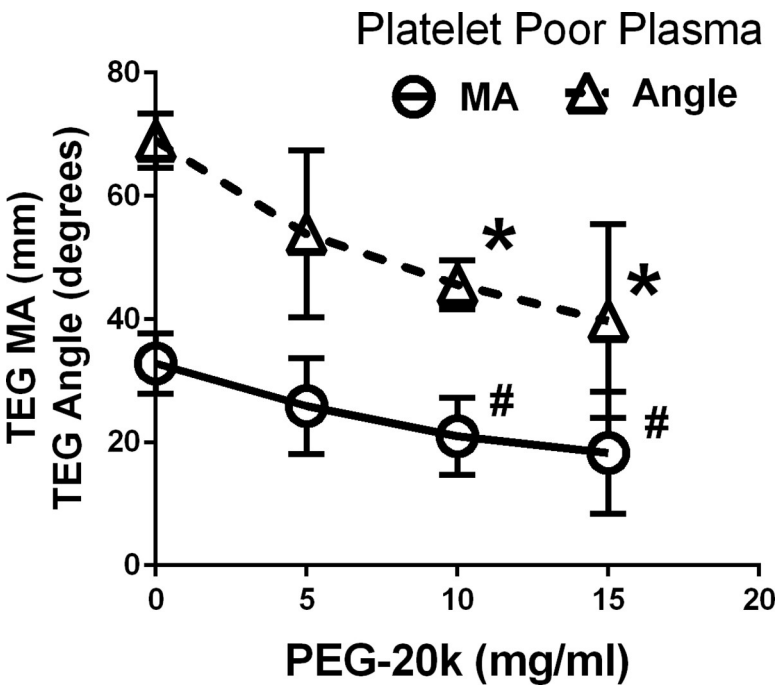

Fig 6. Thromboelastography values of MA and Angle from kaolin activated platelet poor plasma (PPP) obtained from healthy volunteers. All samples were diluted 10% with lactated Ringers solution containing varying concentrations of PEG-20k (0%, 5%, 10%, and 15%). Kaolin activated the PPP. Values represent mean ±SD from fresh blood obtained from 6 volunteers. * and # $P<0.05$, relative to the corresponding PEG-20k dilutional control values (0% PEG-20k).

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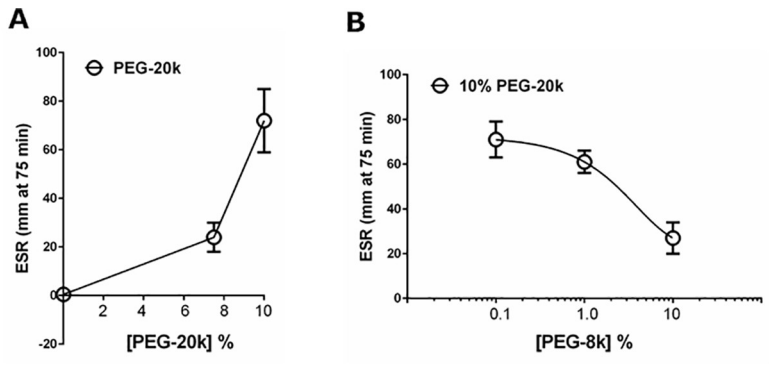

Fig 7. Erythrocyte Sedimentation Rates (ESR) measured in whole blood obtained from healthy volunteers diluted with saline (1 to 9) containing various concentrations of PEG-20k solution (0%, 7.5%, and 10%) (panel A). The dose dependent effects of PEG-8k on the accelerated ESR effect of 10% PEG-20k are shown in panel B. Values are mean +/- SD, n = 4 independent experiments.

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with platelets (80–90%) and fibrinogen (10–20%) [26], it was posited that PEG-20k effects on coagulation may interfere with platelet function and/or fibrin polymerization. Deficiencies in fibrin polymerization or fibrin cross-linking were suspected, given the decreases in the fibrinogen dependent TEG factors such as α-angle and k and changes in clot strength. These changes are affected by low fibrinogen activity, fibrinogen deficiency, Factor XIII defects, or thrombocytopenia/thrombocytopathy. Therefore, to dissect out effects of PEG on coagulation factors or platelet function, we conducted more specific testing on diluted volunteer whole blood.

This study essentially rules out PEG-20k-related effects causing coagulation protein deficiencies. For example, the concentrations of fibrinogen were not different with PEG-20k diluted blood compared to the saline controls, and the fibrinogen remained within the normal ranges. An effect of PEG-20k on fibrinogen shape or charge leading to altered catalytic rates cannot be ruled out and may be a possible avenue to explore in future studies. PEG polymers are known to camouflage active surface molecules on cells [27] so maybe the same can occur with fibrinogen molecules on platelets or in solution. Similarly, the vWF activities were slightly lower in the PEG-20k spiked plasma samples, but also remained in the normal range. Such minor changes likely cannot account for the observed effects on TEG. Therefore, the slower clot propagation and decreases in α-angle and k observed on TEG are probably not the result of lack of plasma fibrinogen or vWF activity. Similarly, since the PT and aPTT times were not different between groups, this suggests both the intrinsic and extrinsic coagulation systems are unaffected by PEG-20k. Although PEG-20k had no effects on aPTT, we observed that activator choices for this test can give a spurious effect. Specifically, the use of an activator containing micronized silica particles to start the intrinsic pathway cascade caused artifactually prolonged clotting times in the presence of PEG-20k. This PEG-silica laboratory interaction has been show previously with PEG per se [28] and with PEGylated factor replacement products [23–25]. The mechanisms for this silica effect are unknown but may be due to a preferential adherence of PEG polymers to the silica, thereby preventing its activation of factors in the intrinsic pathway. Whatever the mechanism, it is important that any future clinical aPTT testing in patients that were given PEG-20k active solutions be tested using kaolin-based activators and not micronized silica activators.

Thrombin generation is an important component of blood clotting and should be evaluated when a coagulopathy is identified since it represents the final common pathway. Furthermore, platelet dependent or independent thrombin activity may be a more important measure of coagulation than PT and aPTT times [19]. Thrombin generation, as indexed by the CAT assay,

indicated a slight but significant decrease in just one measure (Lag Time) of thrombin generation and only in the PRP component of blood diluted with PEG-20k solutions, compared to the saline controls. This small change in thrombin generation was platelet dependent since it was not observed in PPP from the same blood samples. This is consistent with the other platelet-specific changes seen in this study. The contribution of this change in platelet derived thrombin activity, although statistically significant, may not represent a biologically significant factor in the observed effects of PEG-20k on TEG.

The most likely explanation for the slower clot propagation and decreased α-angle and k revolves around the axis of fibrinogen binding to activated platelets. For example, the flow cytometry data showed no difference in platelet receptor expression (PAC1 and CD62P, Fig 5) after ADP activation. However, on TEG platelet mapping, a functional analysis of platelet activation response to ADP and AA, there was a clear unresponsiveness of platelets to stimulation. This may suggest that interference by PEG-20k in platelet clot formation may be downstream from the glycoprotein IIb/IIIa receptor expression after activation. It is tempting to suggest, based on the available evidence to date, that PEG-20k may interfere with IIa/IIIb binding to fibrinogen, thereby interfering with platelet aggregation per se and the amplification of downstream receptor signaling by epinephrine, ADP, collagen, and thromboxanes on platelet aggregation. This is supported by the data showing the MA on platelet mapping and in regular TEG to be reduced with PEG-20k. Furthermore, the lower k and angle values seen with PEG-20k solutions [16], which mimic a functional state of hypofibrinogenemia in the presence of normal fibrinogen concentrations, may be due to blocking of the IIb/IIIa receptor and inhibition of fibrinogen binding and platelet aggregation. Of course, there is also the possibility that PEG-20k directly interferes with the ADP and endoperoxide receptor binding to these specific ligands. Therefore, PEG-20k may induce a state of chemical thrombasthenia at higher concentrations while not significantly affecting the coagulation cascades.

This is further supported, albeit indirectly, by data demonstrating robust effects of PEG-20k solutions on the red blood cell sedimentation rates, which are competitively inhibited by smaller PEG polymers. These data suggest that PEG-20k polymers bind to surface sites on the red blood cell to change their density, possibly through cross linking with other polymer complexes or cell components. If this were to occur in platelets too, then some platelets may be functionally removed from binding with fibrin, fibrinogen, and adhesion molecules to alter the platelet component of clot formation, as documented clearly in our previous study. This proposed parallelism between PEG-20k interactions with RBCs and platelets has not been demonstrated empirically but such a nonspecific passivation effect seems reasonable to postulate from the very strong ESR effects of PEG-20k, and from the known affinity of PEG polymers with cell membrane components, including on platelets [29–32]. Further studies using fluorescent or electron microscopy imaging may be useful to resolve any potential platelet-PEG-20k pharmacodynamic interactions under clot forming conditions. Another potential mechanism explaining PEG-20k on RBC sedimentation may involve shifts in the viscosity of the plasma phase of the blood secondary to unknown PEG-protein interactions, which alters the packing and sedimentation of the RBCs.

Although the MA value derives mostly from platelet function (80–90%), there is still a smaller component (10–20%) attributable to the fibrin component of clot formation. Since PEG-20k was seen to dose dependently interfere with MA on TEG using only platelet poor plasma, we must conclude that a smaller component of the PEG-20k effect on clot strength in clotting whole human blood is due to this fibrin component. The polymer may interfere with activated factor XIII-induced cross-linking and fXIII-fibrin interactions, thereby weakening the clot strength. This is supported by the known interference with other colloids between fXIII and fibrin to limit cross linking and weaken clot strength [33, 34]. Similarly, the platelet

mapping data attributing PEG interferences with ADP and thromboxane-induced platelet aggregation and clot strength could partly be explained by a similar polymer effect on fXIII-induced cross-linking of the reptilase catalyzed fibrin that is formed under platelet mapping conditions (heparin). The exact mechanisms of how PEG polymers may cause these interferences is not known but they may involve a relatively nonspecific passivation of the polymer covering the surfaces of key binding sites on both platelets and fibrin to produce the observed effect on clot formation kinetics and strength.

In conclusion, this study has expanded our search for a mechanistic explanation for the identified effects of PEG-20k solutions on whole blood coagulation observed in healthy volunteers and trauma patients. PEG-20k seems to have less effect on the intrinsic and extrinsic coagulation pathways and on the availability of critical non-catalytic proteins such as fibrinogen and vWF. The effects of PEG-20k solutions on clot formation suggest potential interference by PEG-20k with fibrinogen binding and polymerization on the platelet thereby mimicking a state of mild functional thrombocytopenia, platelet passivation, or thrombasthenia with similar effects on fibrin cross-linking. It may be possible to chemically modify the PEG-20k functional groups to mitigate these effects.

Supporting information

S1 File. Supporting Information.pzf: Original data files and statistical analysis for all figures presented in Prism 6.0 format.
(PZF)

S2 File. Coags-CAT.pzf: Experimental data for the coagulation and CAT experiments, including the statistical analysis and related graphs.
(PZF)

S3 File. Platelet Mapping.pzfx: Experimental data for the platelet mapping experiment including the statistical analysis and the related graphics.
(PZFX)

S4 File. PPP TEG PEG.pzfx: Experimental data, statistical analysis, and related graphs from the thromboelastographic analysis of platelet poor plasma diluted with PEG-20k.
(PZFX)

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Writing – review & editing: Loren K. Liebrecht, Jason Newton, Erika J. Martin, Sudha Jayaraman, Jinfeng Han, Michel Aboutanos, Donald F. Brophy, Martin J. Mangino.

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Acute resuscitation with polyethylene glycol-20k: A thromboelastographic analysis

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BACKGROUND: Previous ex vivo studies have shown that polyethylene glycol-20,000 Da (PEG-20k), a novel synthetic polymer that is highly effective for resuscitation, has a hypocoagulable effect on human blood. This study's objective was to determine the in vivo effects of PEG-20k-based resuscitation solutions on coagulation and platelet function in a porcine model of hemorrhagic shock.

METHODS: Anesthetized pigs underwent controlled hemorrhage until the lactate reached 7 mmol/L or 50% to 55% of their estimated blood volume was removed. A laparotomy was performed to simulate tissue injury. Low volume resuscitation (LVR) was given with fluorescein isothiocyanate-labeled 10% PEG-20k solution (100 mg/mL) or Lactated Ringers, both delivered at volumes equal to 10% of the estimated blood volume (n = 5). Thromboelastography was performed after surgery (baseline), after hemorrhage, and 15 minutes, 120 minutes, and 240 minutes postresuscitation. Hemoglobin was measured to determine changes in plasma volume. Plasma PEG-20k concentration was measured by indicator dilution.

RESULTS: Pigs given PEG-20k survived 2.6-fold longer than controls ($p < 0.001$) and had a significant increase in plasma volume demonstrated by the sustained drop in hemoglobin, relative to controls. Pigs resuscitated with LR died from hypotension an average of 90 minutes after resuscitation compared to the PEG-20k pigs, which all survived 240 minutes and were then euthanized with normal blood pressure and lactate. Administration of PEG-20k primarily decreased the thromboelastograph maximum amplitude, however this began to return toward baseline by 240 minutes. Peak plasma concentration of PEG-20k after LVR were 40% lower than predicted, based on simple dilution (5.7 mg/mL vs. 10 mg/mL) and the half-life was 59.6 minutes.

CONCLUSION: These data demonstrate that acute resuscitation with PEG-20k significantly improves tolerance to hypovolemia but also decreases platelet function in the coagulation cascade, which was due, in part, to its volume expanding effects. (*J Trauma Acute Care Surg.* 2019;87: 322–330. Copyright © 2019 American Association for the Surgery of Trauma.)

KEY WORDS: Polyethylene glycol; hemorrhage; shock; resuscitation; coagulation.

Hemorrhagic shock remains the leading cause of death after trauma in both military and civilian populations, and the choice of resuscitation strategy is an important factor in outcomes.[1–3] Best practices for fluid resuscitation in trauma have shifted away from liberal crystalloid administration to permissive hypotension with small volumes of crystalloid and early use of blood products due to the exacerbation of acidosis, coagulopathy, tissue edema, and trauma-induced capillary leak syndrome caused by crystalloids.[2–5] However, accessing blood products can present a logistical problem in austere, prehospital settings. Furthermore, traditional crystalloids are relatively ineffective since the majority exits the vascular space. Colloids, including starches such as hetastarch, may be more effective at volume expansion, but they are associated with renal toxicity and coagulopathy, and their overall benefit has not been well established.[6–9] Therefore, in the absence of blood products, there continues to be a need for an ideal low-volume resuscitative crystalloid.

Recently, polyethylene glycol polymers have proven to be efficacious in animal models of hemorrhagic shock and resuscitation. Our laboratory demonstrated that polyethylene glycol 20,000 Da (PEG-20k), was highly effective for low volume resuscitation (LVR) in rodent models of hemorrhage, performing several fold better than hetastarch and crystalloid.[10,11] These PEG polymers are cell impermeants that can partially enter the interstitial space and nonenergetically move isotonic fluid from the intracellular and interstitial spaces into the capillaries by osmotic force. This results in lasting volume expansion and decreased metabolic cell swelling, which decompresses the microcirculation. It also improves diffusive and convective capillary oxygen transfer and allows repayment of oxygen debt, lactate clearance, and survival under very low volume conditions.[10–12] These solutions would be ideal for prehospital use when blood is impractical. Furthermore, the improved metabolic and cardiovascular tolerance to shock could allow for much longer transport times without the adverse effects of other fluids.

In a previous ex vivo thromboelastographic analysis using human blood, PEG-20k caused a decrease in clot amplification (K time, α Angle) and decreased clot strength (maximum amplitude

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[MA]). However, when the dose was reduced by 25%, these effects did not occur.[13]

Because in vivo PEG-20k plasma concentration after intravenous infusions are dependent on the algebraic sum of many opposing forces (i.e., blood loss, hemodilution), simple ex vivo coagulation results cannot be extrapolated to a clinically relevant setting.

The objective of this study was to examine the effects of PEG-20k on coagulation and platelet function in an in vivo, preclinical porcine model of severe hemorrhagic shock. A secondary objective was to determine peak plasma concentrations of PEG-20k and establish basic pharmacokinetic parameters. We hypothesized that any coagulation effect of PEG-20k in vivo would be dependent on peak blood levels after resuscitation.

METHODS

All animal experiments were performed under a protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee, which abides by the rules and regulations set forth in the National Institutes of Health and United States Department of Agriculture.

Surgical Preparation

Juvenile, male Yorkshire pigs (30–40 kg, Archer Farms, Darlington, MD) were fasted for 24 hours with free access to water. Anesthesia was induced with ketamine/xylazine/propofol and maintained with 1% to 2% inhalation isoflurane with positive pressure ventilation. Temperature was maintained at 37°C to 38°C. The femoral arteries were cannulated bilaterally for hemodynamic monitoring (Powerlab, ADInstruments, Boston, MA) and for controlled hemorrhage. One femoral vein was cannulated for fluid administration. All vascular catheters were flushed with heparinized saline (5 units/mL with approximately 500 total units of heparin given over 6 hours). A midline abdominal incision was made to simulate soft tissue trauma. Both ureters were cannulated for real time urine collection. A 300-mL bolus of Lactated Ringer's fluid was given to all animals to normalize baseline volume status. After the animals stabilized, baseline vitals were recorded and blood gas analysis was performed to establish baseline biochemical markers, including lactate.

Porcine Shock Model

A previously described porcine model of shock was used.[14] Juvenile pigs underwent controlled hemorrhage via the arterial line, with the mean arterial pressure (MAP) held at 30 mm Hg to 40 mm Hg, until either the lactate reached 7 mmol/L to 8 mmol/L, 112 minutes of hemorrhage time had passed, or 50% to 55% of the animals estimated blood volume (EBV) (calculated using 67 × body weight in kg) was removed. These endpoints were used to ensure a controlled level of oxygen debt across all experiments, based on average values from our previous study.[14] Hemorrhage was initially performed at a rate of 4 mL/kg using a peristaltic roller pump (Masterflex, Cole-Palmer, Chicago, IL) until the MAP dropped to 30 mm Hg to 40 mm Hg and then restarted at a rate of 2 mL/kg once MAP reached 45 mm Hg. During the hemorrhage phase, lactates were measured every 30 minutes using a clinical blood gas analyzer (ABL-800; Radiometer USA, Cleveland, OH). Once one of the three stop triggers above was achieved, hemorrhage was stopped and LVR was given with either a 10% PEG-20k solution (100 mg/mL) in Lactated Ringer's containing a fluorescein isothiocyanate (FITC)-labeled PEG-20k indicator (Nanocs, New York, NY) or Lactated Ringer's (n = 5 in each group) over 5 minutes using the roller pump. In both groups, LVR was given at a volume equal to 10% of the animals EBV. The experiments were stopped either when the animal died or arbitrarily at 240 minutes after resuscitation. The measured LVR Time was defined as the time from the start of LVR to the time the animal died, or when the lactate re-accumulated back to 7 mmol/L to 8 mmol/L. The MAP and lactate value at the end of the experiment were also measured. Hemoglobin concentration over time after LVR was measured as a surrogate measure of plasma volume expansion, using the indicator dilution principle.[12,15] Animals that did not die from hemorrhage and shock were euthanized using intravenous potassium chloride. The shock and resuscitation protocol is shown in Figure 1.

Thromboelastograph Assay

Whole blood thromboelastography (TEG) was used to determine the effect of shock and LVR with PEG-20k on coagulation and platelet function. Thromboelastography was performed after the surgical procedure as a baseline, after hemorrhagic shock just prior to giving LVR, and 15 minutes, 120 minutes, and 240 minutes after resuscitation was given, with a single run for each sample (TEG 5000 Haemoscope; Haemonetics, Braintree, MA). All blood samples were collected in sodium citrate tubes, and the TEG analysis was performed within 30 minutes of blood collection. Clot formation was initiated using re-calcification and kaolin activation with heparinase.

Plasma and Urine PEG Concentration

During the resuscitation period, blood and urine samples were serially collected from animals in the treatment group after LVR was given. The first samples were taken at LVR +15 minutes, or 10 minutes after the completion of the LVR infusion, which was given over 5 minutes, to allow for a steady-state equilibrium to be achieved. The blood samples were spun to isolate plasma, and the concentration of the PEG-20k marker in both plasma and urine was quantified by fluorescence spectroscopy (FL-800; BioTek, Winooski, VT) with an excitation wavelength of 485 nM and an emission wavelength of 520 nM. Plasma concentration data were analyzed by nonlinear regression with a two-phase decay model using GraphPad Prism software (Version 6.7, GraphPad Software, La Jolla, CA). The software was used to determine the terminal half-life and extrapolated time 0 peak concentration.

Statistical Analysis

All statistical analyses were performing using GraphPad InStat for Windows (GraphPad Software, La Jolla, CA; www.graphpad.com). The LVR time, hemodynamic variables, and lactate measurements are expressed as mean ± standard deviation. These data were analyzed using a two-tailed *t* test. The TEG data were analyzed using the Friedman test for nonparametric, repeated-measures analysis of variance with the Dunn's multiple comparisons posttest for data within groups, across time. For comparisons between groups, the Mann

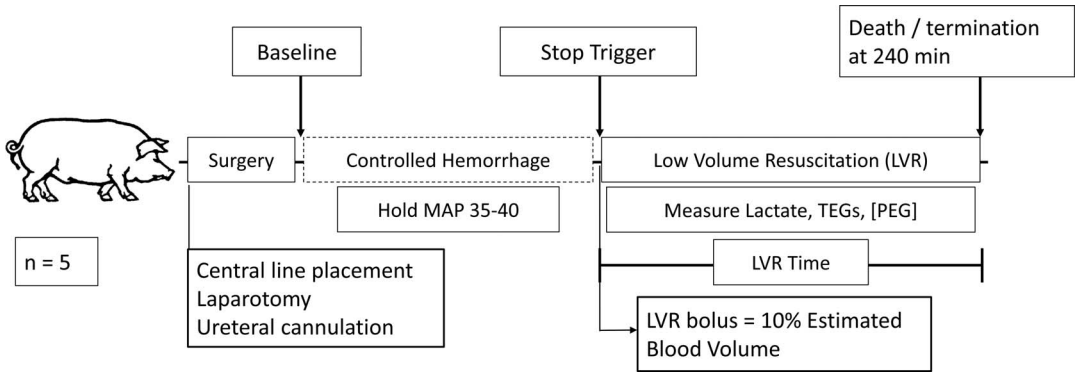

Figure 1. The experimental design of the project using two groups of anesthetized juvenile pigs (n = 5 each). After anesthesia, surgical implantation of vascular catheters is followed by a laparotomy to induce soft tissue trauma. Bilateral ureteral catheters are placed for urine collection. After baseline stabilization (typically 15 minutes), blood gases, laboratories, and TEG samples are obtained. Hemorrhagic shock is induced by blood removal from the arterial line using a roller pump. Blood pressure is held at 35 mm Hg by the pump until one of three triggers is met. These include plasma lactate reaching 7 mmol/L to 8 mmol/L, blood loss of 50% to 55% of total blood volume, or a shock time of 112 minutes. Following the trigger, LVR is administered at a volume of 10% of the EBV, given IV over 5 minutes. Either LR is administered as a volume control or an identical volume of 10% polyethylene glycol 20,000 (PEG-20k) dissolved in LR. After LVR, pigs are monitored until they expire or reach 240 minutes past LVR. Pigs surviving the 240 min protocol are euthanized. Blood gases, blood labs, and TEG analysis are performed serially after baseline, after hemorrhage, and at various times after LVR.

Whitney *U* nonparametric test was used. Finally, the baseline TEG data, prior to shock or resuscitation, from all pigs in the experiment were pooled to create baseline ranges for each parameter because of a lack of normal porcine TEG data. These references are expressed as interquartile ranges (IQR), with n = 10 for all parameters except R, which had n = 5 due to lack of heparinase cups in five of the pigs. A one-sample Wilcoxon signed rank test was used to compare TEG data to the upper and lower limits of the baseline IQR.

RESULTS

Hemorrhage and Resuscitation

The baseline characteristics and hemorrhage parameters did not differ between the two groups, as shown in Table 1. There were no significant differences in the mean baseline weight, blood pressure, or lactate. The hemorrhage volume and time the animals spent in hemorrhagic shock prior to resuscitation were also similar between groups. In both groups, at least 50% of the animal's total blood volume was removed, and the hemorrhagic shock period, or the time from the initiation of bleeding to administration of LVR, lasted 45 minutes to 112 minutes.

PEG-20k increased the LVR time 2.6-fold over the LR control ($p < 0.001$). This was likely an underestimation because all PEG-20k experiments were terminated arbitrarily due to the acute nature of the experiment. In the LR control group, the animals died an average of 90 minutes after resuscitation (range, 49–152 minutes), compared with 240 minutes in the PEG-20k group. The plasma lactate concentrations continued to rise after LR resuscitation but fell to normal after PEG-20k resuscitation and were not significantly different than the baseline values (1.92 mmol/L vs. 2.2 mmol/L, $p = 0.6733$). After LVR with PEG-20k, there was a sustained drop in hemoglobin to an average of 6.5 g/dL from a baseline of 10.3 g/dL ($p < 0.001$). There was no drop in hemoglobin after resuscitation with LR. Given a lack of further bleeding or fluid administration after LVR, the hemoglobin versus time data demonstrate a sustained increase in plasma volume after PEG-20k administration (Fig. 3B). All experiments in the PEG-20k group were all arbitrarily terminated at 240 minutes due to the acute nature of the experiments.

TABLE 1. Characteristics of Both Treatment Groups During Hemorrhage and Resuscitation

Hemorrhage/Resuscitation	Control: LR	Treatment: 10% PEG-20k
Weight (kg)	34.32 (5.12)	29.4 (2.23)
EBV (mL)	2299.4 (342.3)	1969.5 (150)
Baseline MAP (mm Hg)	79.6 (10.5)	70.8 (3.7)
Baseline lactate (mmol/L)	1.9 (0.46)	1.92 (0.64)
Baseline hemoglobin (g/dL)	10.6 (0.44)	10.3 (0.74)
Hemorrhagic shock time (min)	62.6 (18.3)	74.8 (34.5)
Hemorrhage volume (mL/%)	1196/52% (158/2.9%)	1036.5/53% (131/3.4%)
LVR volume (mL)	229.6 (34.2)	197 (15)
LVR time (min)	90.8 (42)	240 (0)*
Terminal lactate (mmol/L)	12.6 (2.9)	2.2 (1.2)*
Terminal MAP (mm Hg)	N/A**	56.6 (5.9)

TEG baseline reference values	Median (IQR)
R (min)	4.8 (3.3–5.1)
K (min)	1.5 (1.13–1.78)
Angle (degrees)	71.65 (68.3–74.3)
MA (mm)	72 (70.0–77.8)
LY30 (%)	2.05 (1.63–2.4)
CI	2.1 (−0.03–3.78)

Continuous data expressed as means (standard deviation).
*$p < 0.001$ | **No terminal MAP data—animals expired.
TEG reference values: n = 5 for R, n = 10 for all other parameters.

Coagulation

The coagulation and platelet function data are presented in Figures 2–4. At the baseline (BL) time point, the R value data in the LR groups is not shown due to inaccuracy resulting from a lack of heparinase cups used for some of these samples (Fig. 2A). Analysis of the other parameters, K time, angle, MA (Fig. 2B–D) and the Coagulation Index (Fig. 3A), demonstrates that there were no significant differences between the PEG-20k group and LR controls at the BL time point and after hemorrhagic shock (HS). The baseline data from both groups was pooled to create baseline ranges for each parameter. Table 1 lists the medians and interquartile ranges for each parameter.

Hemorrhage and surgical trauma caused a trend toward increased clotting, however, these changes were not statistically significant for each individual TEG parameter when comparing the posthemorrhage data to baseline. For instance, after hemorrhage (HS), the mean R time decreased, the K time decreased, the Angle increased, the MA increased, and the Coagulation Index increased from the BL in both groups. Again, these changes did not achieve statistical significance.

After LVR was given, there were immediate effects on TEG when comparing the postresuscitation data across time (LVR 15–LVR 240) to the HS time point within the PEG-20k group (Fig. 2). Specifically, administration of PEG-20k caused a decrease in the MA from a posthemorrhage (HS) mean of 70.64 ± 4.689 mm to a mean of 58.1 ± 4.794 mm at the LVR + 15-minute time point ($p < 0.05$). Using the baseline interquartile range as the reference value in this experiment, MA was reduced below its lower limit (IQR: 70.0–77.8 mm) at this time point. While PEG-20k did not cause statistically significant changes to the other TEG parameters at the 15 minute time point, the Coagulation Index (CI) (Fig. 3) did decrease from 3.38 ± 1.163 posthemorrhage (HS) to 0.1 ± 0.383 at LVR + 15 minutes ($p < 0.05$). However, both the CI and the parameters R, K and Angle remained within their baseline reference ranges at the LVR +15 minute time point. TEG analysis at later time points during the experiment revealed effects on the R time, K time, and Angle. When comparing the HS time point to LVR + 240 minutes within the PEG-20k group, there was an increase in the R time and K time and a decrease in the Angle ($p < 0.05$), and all of these were outside of their respective baseline reference ranges. However, by LVR + 120 minutes, the MA began to recover from the initial effect of PEG and although the values fell below the lower reference limit, they were no longer significantly different than the posthemorrhage or baseline values (Fig. 2D).

In the LR control group, data are only available through the LVR + 15 minute time point because all but one animal expired before 120 minutes. While all the parameters continued to trend towards a hypercoagulable profile at LVR + 15 minutes, none were statistically different than their corresponding

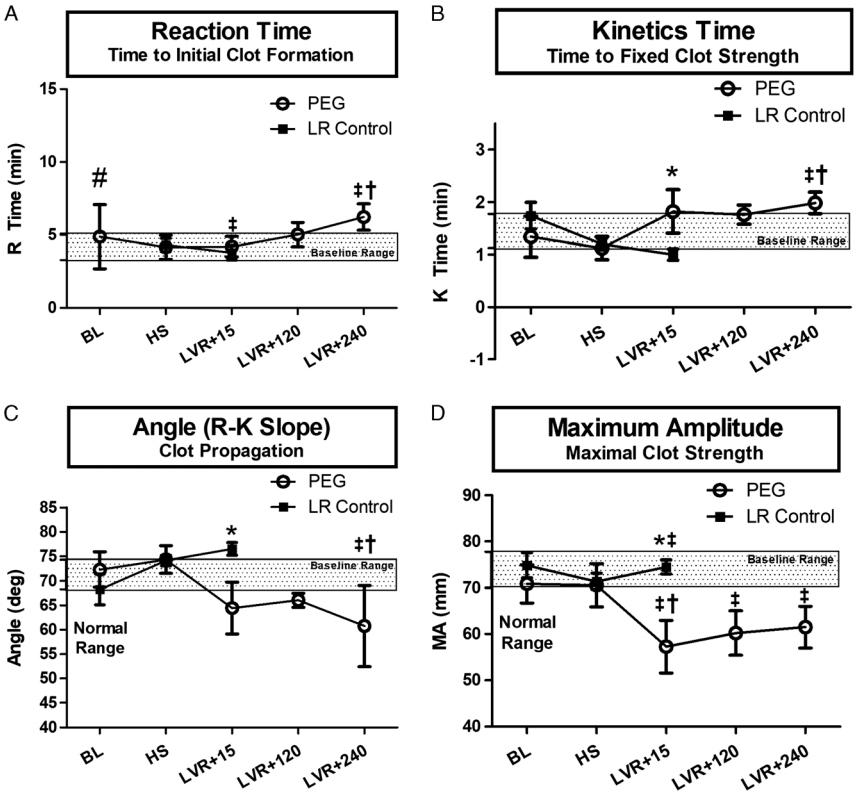

Figure 2. TEG values for R time (A), K time (B), angle (C), and MA (D) are shown for the two groups of pigs (LR, black squares and PEG-treated, open circles). The blood samples from each pig in both groups were analyzed at BL, after HS, and after LVR for 15 minutes, 120 minutes, and 240 minutes. Values are expressed as mean ± standard deviation, n = 5, † $p < 0.05$ compared to the HS time point in the same group,* $p < 0.05$ compared to the other group at the corresponding time point, ‡ $p < 0.05$ compared to baseline range; # R data in the LR group at baseline was excluded due to inaccuracy. The hatched area represents the baseline reference values for the various TEG parameters.

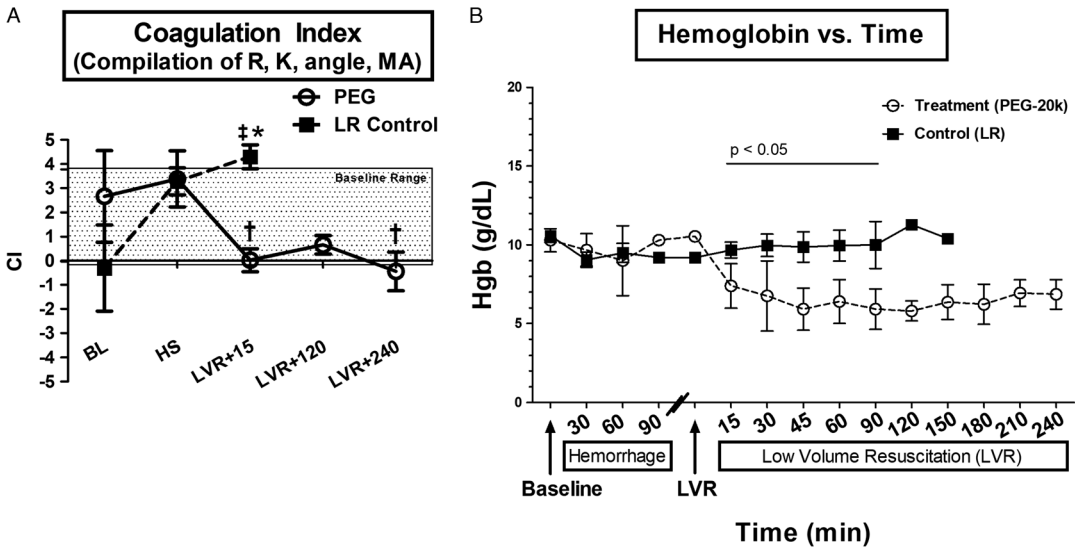

Figure 3. TEG values for the CI (A) and hemoglobin concentration (B) are shown for the two groups of pigs (LR, black squares and PEG treated, open circles). The coagulation index is a mathematical model of the overall coagulation state using values from R, K, angle, and MA. The blood samples from each pig in both groups were analyzed at BL, after HS, and after LVR for 15 minutes, 120 minutes, and 240 minutes. Values are expressed as mean ± standard deviation, n = 5, † $p < 0.05$ compared to the HS time point in the same group,* $p < 0.05$ compared to the other group at the corresponding time point, ‡ $p < 0.05$ compared to baseline range. The hatched area represents the baseline reference values for the various TEG parameters. Hemoglobin versus time demonstrates the plasma volume expansion and dilution of clotting factors and platelets that occurred after LVR with PEG-20k.

posthemorrhage, pre-LVR values (HS). Resuscitation with LR did increase the Angle above its upper limit (IQR, 68.3–74.3 degrees) to 76.5 degrees at the LVR + 15-minute time point. When comparing PEG-20k and LR at the LVR + 15-minute time point, the PEG-20k group had a significantly higher K time (1.82 vs. 1.0, $p = 0.01$), a lower angle (64.62 vs. 76.5, $p = 0.0082$), and a lower MA (58.1 vs. 75.4, $p = 0.005$), all of which resulted in a decreased Coagulation Index (0.1 vs. 4.3, $p = 0.008$). Representative TEG tracings from each group at various time points are shown in Figure 4.

There were no statistical differences in the fibrinolysis (LY30) data when comparing groups at each time point, when analyzing within-group repeated measures, or when comparing each time point to the upper limit of the baseline range. However, in the PEG-20k group, there was a trend toward increasing fibrinolysis as the experiment continued, with an LY30 of 4.02% at LVR + 240 minutes versus 2.54% at baseline ($p = 0.3644$). The LVR + 240 LY30 value was higher than the upper limit of the baseline range (2.4%), but did not achieve statistical significance, with $p = 0.0625$.

Plasma and Urine PEG Concentrations

The plasma and urine PEG concentration data are displayed in Figure 5. The plasma concentration of PEG-20k

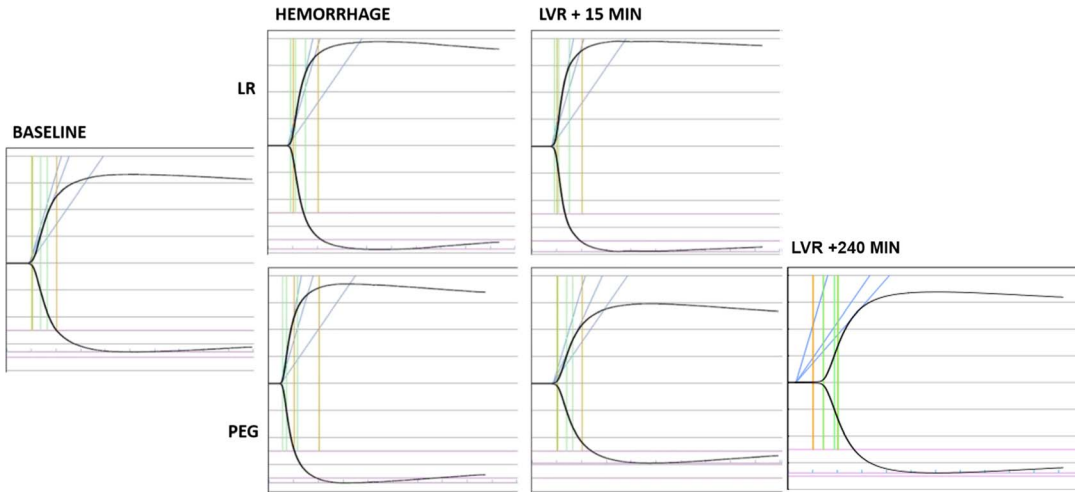

Figure 4. Representative TEG tracings from whole blood obtained from pigs in the LR group and the PEG-20k group measured at baseline, after hemorrhage (right before resuscitation), 15 minutes and 240 minutes after LVR (+15 minutes, LVR + 240).

was 3.58 mg/mL 15 minutes after administration. The data were analyzed using nonlinear regression using a two-phase exponential decay model. The R^2 was 0.99, indicating an adequate goodness-of-fit. The model-derived termination half-life of PEG-20k was 59.6 minutes, and the extrapolated time zero peak plasma concentration was 5.7 mg/mL (Fig. 5A). Similarly, PEG-20k appeared in the urine within 15 minutes of administration and followed a similar decay pattern as the plasma concentration (Fig. 5B).

DISCUSSION

The purpose of this study was to determine the effects of LVR with PEG-20k on coagulation in a clinically relevant model of controlled hemorrhagic shock, mimicking the military and prehospital settings where blood is not routinely available. The data demonstrated that when compared with the vehicle control (LR), PEG-20k significantly improved survival and hemodynamic parameters. PEG-20k did affect coagulation, most significantly decreasing the MA compared to the within group baseline and to the LR control. Although the MA remained outside of the baseline reference range, it approached its preresuscitation value by the end of the experiment, suggesting resolution of this effect.

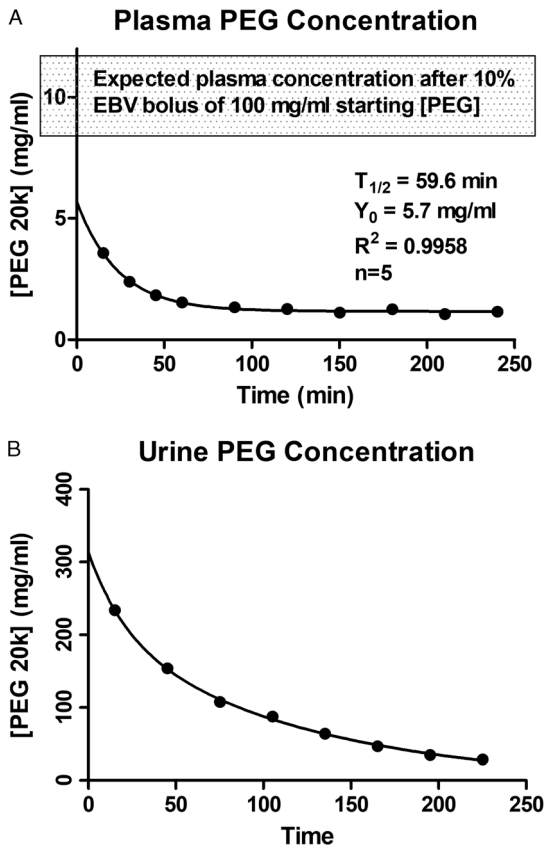

Figure 5. Kinetic analysis of the disappearance of plasma and urine concentrations of PEG-20k after intravenous infusion of the PEG-based LVR solutions after hemorrhage. The 10% PEG-20k LVR solutions were spiked with a FITC-labeled PEG-20k tracer molecule, which was detected in the plasma and urine using spectrofluorometry. Disappearance curves were then constructed to determine the peak plasma concentrations after LVR and the effective plasma half-life of elimination, with an extrapolated peak PEG-20k concentration of 5.7 mg/mL at time 0. The rapid clearance of PEG-20k can be seen by the rapid appearance and disappearance of FITC-PEG in the urine, which is the major route of elimination of the polymer. All data points represent the average of five independent pig studies.

The basis for the current study was a previous experiment by our group in which human whole blood was diluted ex vivo with a 10% volume dose of the PEG-20k solution (100 mg/mL) and analyzed using TEG.[13] The ex vivo 10% volume dilution was chosen because it mirrored the volume administered in our animal models of hemorrhagic shock, representing the highest possible range of PEG-20k dosing for low-volume resuscitation. The ex vivo data showed that the 10% dose of PEG-20k significantly affected both clot propagation (increased K time, decreased Angle) and clot strength (decreased MA). However, these effects were neutralized when the dose was reduced to a 7.5% volume dilution.[13] The ex vivo assay differs from the in vivo study described here in two important ways. First, it cannot reproduce the effects of the endothelial surface on the coagulation system. And, second, an ex vivo design cannot account for factors that affect the plasma levels of PEG-20k, such as the amount of blood loss, dilution and distribution of PEG, and clearance.

When analyzing the in vivo data in the context of this previous ex vivo study, we considered the LVR + 15 minute time point to be the most relevant since it allowed enough time for the molecule to reach steady state. There was a parallel decrease in the MA in this study, again suggesting the PEG-20k may interfere with platelet activity and clot strength. However, the Angle and K time were not as significantly affected compared to the ex vivo study, and they were not statistically different than the baseline reference limits at the 15-minute time point. Furthermore, as the experiment continued, the MA returned towards its baseline value in the PEG group, and the TEG tracings shown in Figure 4 demonstrate this qualitatively as the overall shape of the curve at LVR 240 returns to the baseline shape. This was most likely due the difference in blood levels when the solution was administered IV compared with the theoretical 10% dilution assumed in the ex vivo whole blood tests. In fact, the extrapolated peak blood level from the regression was 5.7 mg/mL, or about 60% of 10 mg/mL, which was the minimum expected plasma concentration when giving a 100-mg/mL PEG-20k solution at a volume equal to 10% of the total blood volume prior to any blood loss. This data supports our hypothesis that the effect of PEG-20k on coagulation is dependent on plasma concentration.

This large discrepancy in blood levels may be due to a misunderstanding of the forces that distribute and dilute the polymer in the circulation. The net dilutional effects are the algebraic sum of the forces that tend to dilute the polymer minus those that tend to concentrate it. Specifically, PEG-20k is diluted by its distribution to water soluble compartments outside of the intravascular space because about one third of the intravascular PEG-20k moves into the interstitial space, leaving only 67% in the blood compartment.[10] Furthermore, the PEG-20k molecules left behind in the blood compartment are further diluted by the oncotic movement of water into the vascular space. Finally, a large amount of material seems to be rapidly excreted into the urine. On the other side of the equation, a predominant factor

that may serve to raise PEG-20k blood concentrations in this model (above the theoretical dilution value) would be the 50% blood loss from the severe hemorrhage. Clearly, the net forces favor dilution of the polymer above what was estimated.

The effect of PEG-20k on the MA diminished over time, suggesting that PEG-20k causes a reversible, functional thrombasthenia. The exact mechanism of PEG-20k's effect on platelet function cannot be determined using TEG alone, but one hypothesis is that it physically passivates platelets via adsorption. Although PEG itself is generally considered to be inert, its structure attracts shells of water molecules, creating a large exclusion volume that can potentially camouflage the surface receptors on platelets.[16–18] This would also explain the effects of PEG-20k on ex vivo clot propagation, which may be due to decreased access to the catalytic surface on the platelet membrane. In addition, we found that PEG-20k caused late effects on the TEG parameters representing enzymatic factors and fibrinogen – R, K and Angle – at the LVR +120 minutes and +240 minutes time points in this experiment. While we suspect this is partly due to hemodilution of clotting factors given the large amount of volume expansion demonstrated by indicator dilution of hemoglobin over time (Fig. 3B), further mechanistic studies are required to delineate the exact mechanisms of PEG's effects on clotting factors and platelets.

The finding in this study that PEG-20k had sustained hemodynamic effects despite rapid clearance in the urine may warrant further investigation. It is possible that much lower plasma levels of PEG-20k can be clinically effective, although when a reduced dosed was studied in rats, it was less effective for hemorrhagic shock than the 10% EBV dose.[10] Another possibility is that by maintaining oxygen transfer early after shock, PEG-20k prevents the vicious cycle of microvascular injury leading to more tissue-level ischemia and vice versa. Specifically, there is literature regarding the use of PEG in preservation solutions for organ transplantation which shows that it prevents ischemia/reperfusion injury.[19–21] Since severe hemorrhagic shock is essentially ischemia at the tissue level, biochemical assays of ischemia/reperfusion injury and intravital microscopy of the microcirculation may be of use in investigating this potential mechanism of action.

The major limitation of this study is that controlling for scientific comparisons limits its translatability to unpredictable, real-world trauma settings. We used a pure vehicle (placebo) control of LR and a controlled hemorrhage model of shock without poly-trauma to ensure that the effects on TEG could be attributed to PEG-20k alone, and not to further blood loss or other factors, and to allow accurate pharmacokinetic determinations. Clinically, this would be similar to patients with extremity bleeding that has been controlled in the field with a tourniquet. This study supports the idea that PEG-20k would be able to extend the upper limit of transport times prior to definitive resuscitation with blood and surgical treatment for this group of patients. Future directions include assessing the efficacy of PEG-20k in models of uncontrolled hemorrhage, poly-trauma, and survival using clinically relevant controls, such as higher volumes of crystalloid, starches, or shelf-stable and fresh blood products. Another limitation of this study involves the idiosyncrasies of TEG and the fact that normal values in pigs are different than humans.[22] Because TEG is influenced by a number of factors including activation method, time to running the sample, and arterial versus venous sampling, and so on,[23–25] we elected to create "normal" reference ranges using the baseline data from all animals for subsequent comparison. Ideally, normal ranges for pigs should be determined using a larger sample size.

In summary, this study demonstrated that when given at doses that are highly effective for LVR of severe hemorrhagic shock, PEG-20k had a significant effect on MA, suggesting an interaction with platelets. This effect began to wane at the end of the acute phase, suggesting a reversible mechanism. These effects were small and, although they were statistically significant, they may well also be clinically irrelevant. Pharmacokinetic data demonstrated a lower than expected peak plasma concentration and rapid clearance, which is responsible for reduced effect of PEG-20k compared to prior ex vivo TEG analysis. Further studies are required to elucidate the mechanism of PEG-20k's effect on coagulation and platelet activity.

AUTHORSHIP

Each author has contributed significantly to and is willing to take public responsibility for aspects of this study. N.W., M.A., and M.M. contributed to the literature review study design. N.W., K.K., H.R., C.B., R.L., M.A., and M.M. performed the data acquisition. N.W. and M.M. performed the analysis and interpretation of data and wrote the article.

DISCLOSURE

The authors declare no conflicts of interest.

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DISCUSSION

JEREMY W. CANNON, M.D. (Philadelphia, Pennsylvania): Dr. Agarwal, Dr. deMoya, members and guests, thank you for the opportunity to discuss this manuscript that was very well presented by Dr. Wickramaratne.

This preclinical, large animal study from the Mangino laboratory evaluates a novel resuscitation fluid. This fluid, PEG-20K, is based upon the familiar polyethylene glycol molecule that not only has a well-established track record as an osmotic laxative – think Miralax or GoLytely – but also has emerging applications in the nanoparticle realm.

PEG-20K is appealing as a resuscitation fluid for prehospital and prolonged field care use because it has shelf stable properties and seems to be effective in very small volumes. It really does "pack a punch."

So what can we take away from the results we just saw? The survival findings mirror the rodent experiments that were published by this same group. PEG-20K appears to improve tolerance to low-volume resuscitation.

Indeed, while none of the lactated Ringer's animals survived the four-hour resuscitation period, all of the PEG-20K animals made it to the experimental endpoint.

So what about the effect on coagulation? Using TEG these authors found that PEG-20K, even in small volumes and low concentrations, has a very rapid and I would say negative effect on clot strength, as well as decreased fibrin crosslinking that became significant at four hours. So although the PEG-20K animals did survive, their platelet function was significantly impaired at even low doses of this fluid.

The authors contend that PEG-20K took these animals from a state of hyper-coagulation after hemorrhage and simply normalized the situation. But I have some concerns over this framing of the results – most significantly, the normal ranges presented are for human data.

We know from data published by the Denver group that fibrin crosslinking and clot strength is much higher in pigs than in humans. So these normal ranges should be shifted significantly before drawing any conclusions about the degree of coagulopathy induced by this resuscitation fluid.

In that light, I have a couple of questions for the authors.

First, why did you choose such a lethal model? Because it is so lethal, your model unfortunately precludes the ability to characterize the native coagulopathy over time in negative control animals after a hemorrhagic insult.

Second, why did you use human normal reference ranges for comparison? I believe based on what we know about porcine coagulation this is misleading.

Third, in your prior rodent experiments you actually included a hetastarch arm. As you indicated, hetastarch really is a great comparison solution to PEG-20K. Why was this not done in the present study?

And, finally, because the coagulopathy effects of PEG-20K seem to be concentration-dependent, there actually may be a "sweet spot" where both survival and normal coagulation function are possible at lower doses. Could you please comment.

Alternatively, this therapy could be combined with hemostatic treatments such as TXA or dried plasma. Have you explored any of these various possibilities?

Congratulations on a well-conducted study and thank you to the Association for the privilege of the podium.

NILUKA A. WICKRAMARATNE (Richmond, Virginia): Thank you for the questions, Dr. Cannon. So to answer your first question, why such a lethal model?

I think the impetus for that was our drive to create something that can really increase transport times for the military in the setting of severe hemorrhage. So we were working within that context in a model that we had used already.

I agree it definitely precludes comparison to other resuscitation strategies and something we want to do is include a hetastarch arm and not just low-volume resuscitation but a hetastarch arm that can allow for survival and a saline or control, or an LR control arm that can allow for survival out that long for comparison data.

Historically, we haven't been able to get our animals to survive with hetastarch given at those low volumes so that's why we didn't use an arm with that in the first place.

Thank you for your comments about the human ranges. I also looked at it with regards to our own baseline ranges in

pigs, before the hemorrhage period, which is reflected on the graph today.

And it's definitely higher than in humans for the MA and for the angle. But at the end of the experiment we found that the MA in the PEG treatment group actually lost any significance in terms of difference from the baseline values, which corresponded to PEG being cleared by the kidneys, with even further reduced concentrations in the plasma, and further reduction of its effect on the coagulation system.

In terms of lower doses to avoid those effects, we have found that the 10 percent blood volume dose is the most effective in terms of survival or LVR time.

In a rodent study we reduced the does to a 7.5 percent initial IV bolus and the animals didn't survive as long. Now whether that would translate to pigs or not is a question to be answered.

And definitely combining it with medications that could combat the effects on coagulation would be a good strategy and something we would like to look at.

Superior Survival Outcomes of Polyethylene Glycol-20k Resuscitation Solution in a Preclinical Porcine Model of Lethal Hemorrhagic Shock

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Running Head: Novel polymer based crystalloid resuscitation for hypovolemia

MINI-ABSTRACT

Low-volume resuscitation with the new crystalloid polyethylene glycol 20,000 (PEG-20k) in a porcine model of lethal hemorrhagic shock significantly increased 24-hour survival when compared to resuscitation with Hextend or fresh autologous whole blood (survival =100%, 0%, and 17%, respectively). Secondary metabolic, cardiovascular, and neurological outcomes were also significantly improved with PEG-20k resuscitation. These data realign our thinking about the importance of tissue capillary perfusion compared to oxygen carrying capacity in shock resuscitation.

ABSTRACT

Objective: To compare early outcomes and 24-hour survival after low-volume resuscitation (LVR) with the novel polyethylene glycol-20k-based crystalloid (PEG-20k), whole-blood (WB), and Hextend in a preclinical model of lethal hemorrhagic shock (HS).

Background: Posttraumatic HS is a major cause of preventable death. Current resuscitation strategies focus on restoring oxygen-carrying capacity (OCC) and coagulation with blood products. Our lab showed that PEG-20k is an effective non-sanguineous low volume resuscitation (LVR) solution in acute models of HS through mechanisms targeting cell swelling-induced microcirculatory failure.

Methods: Pigs underwent splenectomy followed by controlled hemorrhage until lactate reached 7.5-8.5 mmol/L. They were then randomized to receive LVR with PEG-20k, WB, or Hextend. Surviving animals were recovered four hours post-LVR. Outcomes included 24-hour survival rates, MAP, lactate, hemoglobin, and estimated intravascular volume changes.

Results: Twenty-four-hour survival rates were 100%, 16.7%, and 0% in the PEG-20k, WB and Hextend groups, respectively (p=.001). PEG-20k produced significantly higher MAP and restored intravascular volume to baseline levels, compared to the other groups. This resulted in complete lactate clearance within four hours despite decreased OCC. Neurological function was normal after next-day recovery in PEG-20k resuscitated pigs.

Conclusion: In a preclinical porcine model of lethal HS, superior early and 24-hour outcomes were observed with PEG-20k LVR compared to WB and Hextend, despite decreased OCC from substantial volume-expansion. These findings, if reproduced in future clinical trials, should prompt re-evaluation of early resuscitation strategies with emphasis on microcirculatory perfusion.

INTRODUCTION

Traumatic injury is a major global cause of death in all age groups and especially in young adults.(1, 2). In the United States, more than 2.3 million deaths were ascribed to unintentional injuries between 1999 and 2017, and an alarming trend of increased incidence of trauma-related mortality has been seen in recent years.(2, 3) Importantly, posttraumatic hemorrhagic shock (HS) is the leading cause of preventable death in civilian and combat trauma with the majority occurring before reaching surgical or critical care facilities.(4-6). Traditional resuscitation strategies based on aggressive crystalloid administration have now been largely replaced by the concepts opf permissive hypotension and low-volume resuscitation (LVR), with early administration of blood products as soon as available. This relatively recent paradigm is sometimes referred to as damage control resuscitation. In addition to restoring oxygen carrying capacity (OCC) and coagulation factors with blood components, crystalloid minimization reduces the risk of its associated triad of acidosis, dilutional coagulopathy, and hypothermia that can exacerbate uncontrolled hemorrhage or subsequently cause acute respiratory distress syndrome, abdominal compartment syndrome, and/or anasarca.(7-10). The current ‘gold standard’ involves a balanced transfusion of red blood cells (RBCs), plasma, and platelets, at equivalent ratios to mimic the transfusion of whole blood (WB). While prehospital blood transfusion has been shown to carry survival benefits (11, 12), logistic constraints often limit the availability of blood products soon after injury when early effective resuscitation is key to survival (4, 5, 13). Furthermore, low volume crystalloid solutions containing hypertonic saline, hydroxyethyl starch, or other polymers as oncotic volume expanders, have shown suboptimal clinical results (14-16). Consequently, a clamant need remains for a new solution that is temperature-stable, transportable, and effective in relatively low volumes and can be safely administered in the prehospital setting.

Of significance, understanding the causal pathophysiology of shock and resuscitation injury may help identify potential targets for treatment. After traumatic HS, inadequate tissue oxygenation and loss of cellular bioenergetics reduce energy-dependent cell volume control mechanisms including the NA^+/K^+ ATPase pumps. This results in cellular swelling that compresses the microcirculation, which further hinders organ perfusion and exacerbates ischemic injury. The resulting 'no-reflow' phenomenon is further exaggerated by crystalloid overloading with sodium and water (17-22).

In recent years, our lab has demonstrated that a novel solution of isotonic crystalloid [lactated Ringer's (LR)] containing 10% polyethylene glycol with a molecular mass of 20,000 Dalton (PEG-20k) is very effective for LVR in acute animal models of severe HS. Owing to unique molecular properties (23), PEG-20k exerts a hybrid impermeant-oncotic effect targeting the ischemia-related cell-swelling by moving isotonic fluids out of the cells into the interstitium then to the intravascular compartment. This decompresses the microcirculation, reloads the capillaries, and restores local blood flow in an otherwise severely shocked state (17, 18, 22, 23). We previously reported superior outcomes of LVR with PEG-20k compared to saline and LR (crystalloids), and to albumin and hextend (colloids) in acute rodent and/or porcine models of HS (18, 23, 24). PEG-20k was associated with manyfold improvements in tolerance to the low volume state with significant reductions in oxygen debt despite sizable decreases in OCC caused by auto-hemodilution. In light of these findings, we raised the question whether restoring OCC with blood transfusion should remain the major priority of early resuscitation strategies or whether prompt protection of the microcirculation from the detrimental cascade of exchange failure (caused by metabolic cell-swelling) should be the primary target in the early stages of shock treatment. Additionally, evidence is still lacking on whether or not LVR solutions targeting this mechanism are sufficient for survival beyond a few hours (of low hemoglobin levels), without definitive resuscitation with blood. This is of paramount importance in the prehospital setting with prolonged field care, extended transport time, and in the

unusual but dire case of mass casualties (25). Therefore, the aim of this study was to compare the early post-resuscitation and the 24-hour outcomes of LVR with PEG-20k, whole blood (WB), or Hextend, in a preclinical large animal model of life-threatening HS and soft tissue injury.

METHODS

Animals and Surgical Preparation: This study was approved by the Virginia Commonwealth University's Institutional Animal Care and Use Committee. The experimental design is depicted in Figure 1. After 16-20 hours of fasting, 17 male Yorkshire pigs (Archer Farms, Darlington, MD) weighing 34.8 ± 3.1 kg were sedated with intramuscular ketamine (20 mg/kg) with xylazine (2 mg/kg) followed by anesthesia induction using intravenous propofol (2-3 mg/kg). Anesthesia was maintained with isoflurane at 1% to 2% in room air (FiO2 of 0.21) while on mechanical ventilation adjusted to an end tidal CO_2 of about 40 mm Hg. A circulating water-warming pad was used to maintain normothermia. Both superficial femoral arteries were cannulated for blood pressure and heart rate monitoring (PowerLab, ADInstruments inc., Dunedin, New Zealand) and for controlled arterial hemorrhage. Additionally, the external jugular vein was cannulated for fluid administration. A laparotomy and splenectomy were performed to produce soft tissue and organ injury and to mitigate the effect of autotransfusion of stored RBCs in the spleen.

Hemorrhagic Shock Model: An intravenous fluid loading bolus of LR solution (10 mL/kg) was administered over 10 minutes before baseline data and labs were obtained. Controlled hemorrhage was then initiated by arterial bleeding at a 2 ml/kg rate using a Masterflex® peristaltic roller pump (Cole-Parmer, Chicago, IL) until mean arterial pressure (MAP) reached 35-40 mmHg. After allowing the animal to compensate to a MAP of 45-50 mmHg, bleeding was resumed until one of the two

shock endpoints was reached: 1.) A plasma lactate of 7.5-8.5 mmol/L was reached within 112 minutes of hemorrhage time and under a total hemorrhage volume of 53% (TBV) or 2.) Both hemorrhage time and hemorrhage volume limits were met without achieving the lactate goal (24).

Low-volume Resuscitation and Study Outcomes: Once the shock endpoint was reached, the animals were randomized to receive an intravenous bolus (over 5 minutes) equal to 10% TBV (LVR) of either of PEG-20k (n=6), WB (n=6), or Hextend (n=5). During the controlled hemorrhage, blood was stored in a Viaflex plastic bag containing sodium citrate and was used for resuscitation in the animals randomized to the WB group (autologous blood transfusion). Vital signs were recorded and lactate and hemoglobin levels were measured every 15 minutes after LVR. The experiment was terminated and the animals euthanized when MAP consistently dropped below 30 mmHg. If the pigs survived for 240 minutes after LVR, they were recovered from anesthesia. Surviving animals were weaned off anesthesia, recovered, provided post-operative analgesics (Buprenorphine SR), and allowed access to food and water. On postoperative day 1 (POD1), a 24-hour neurologic assessment score was determined and the pigs were taken back to the operating room for a terminal data and specimen recovery procedure.

The primary outcomes of the study were the 24-hour survival rates and NDS. Secondary outcomes included: MAP, plasma lactate concentrations, and hemoglobin values. Neurologic function on POD1 was evaluated using a standardized scoring that considers behavior and level of consciousness, breathing pattern, cranial nerve function, and motor and sensory function. An NDS of 0–40 is considered as absence of neurologic deficit, a NDS of 400 as brain death (26). Finally, an indicator dilution method of hemoglobin was used to estimate changes in intravascular compartment volume after LVR assuming no further blood loss was allowed after reaching the controlled hemorrhage endpoint (Supplemental Figure 1). Different hemodilution-based techniques have been

used by others to calculate intravascular volumes in various clinical and experimental settings (27, 28) and we have previously validated the use of hematocrit changes against fluorescein isothiocyanate (FITC)-labeled albumin in a rat model of controlled HS (18).

Statistical Analysis

Data are expressed as means ± standard deviation (SD). IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA) was used for Statistical analysis. Comparisons between the 3 groups were performed with Fisher's exact test (for categorical variables) and One-way ANOVA with Bonferroni multiple comparison correction (for continuous variables). Paired comparisons within each group were performed using paired t-tests. Statistical significance was determined if $p \leq 0.05$.

RESULTS

At baseline, animals in all groups had comparable weigh, MAP, lactate, hematocrit, creatinine and other measures of organ function (table 1). The overall mean % hemorrhage (of TBV) was 47.7 ± 6.5 which resulted in an elevation of plasma lactate to 7.9 ± 1.1 mmol/L over an average shock time of 71.7 ± 27.8 minutes. There were no differences between groups in any of these shock parameters before the start of LVR (Figure 2).

All animals (100%) in the PEG-20k group survived 24 hours compared to only one (16.7%) in the WB group and none (0%) in the Hextend group (p=0.001). Consequently, mean survival time was substantially longer in the PEG-20k group (24 hours) compared to the 2 other groups ($p < 0.001$ for both comparisons, while it did not differ statistically between the WB and Hextend (5.2 ± 9.2 vs 2.2 ± 1.4 hours, respectively, p=0.097). The NDS of the one surviving pig in the WB group indicated

moderate disability whereas all PEG-20k-resuscitated animals had a normal NDS (Table 1 and Figure 3).

Following resuscitation, MAP increased to higher levels with PEG-20k compared to WB and Hextend ($p < 0.05$). These significant differences were observed throughout the acute phase of the experiment (LVR-15 to LVR-240). Again, no statistical differences were observed in blood pressure between the WB and Hextend groups (Figure 4). LVR with PEG-20k resulted in a complete lactate clearance by the LVR-240 time point. Normal lactate was maintained for 24-hours despite a decrease in MAP from 75.8 ± 9.1 at LVR-240 to 61.3 ± 9.1 mmHg at POD1 ($p = 0.039$). Lactate at LVR-240 (terminal) was 2.9 ± 1 mmol/L which was not significantly different than baseline or POD1 levels. The one animal that survived 24 hours in the WB group had a terminal lactate level of 7.4 mmol/L. Lactate clearance, as well as MAP improvement, did not continue in the animals resuscitated with WB and Hextend beyond 1-2 hours (with the exception of the one pig that survived 24 hours in the WB group) and terminal lactate was not different between the 2 groups (11.3 ± 3.1 and 12.5 ± 4.7, respectively) but significantly higher than the PEG-20k group (2.9 ± 1 mmol/L $p = 0.001$).

Significant reduction in hemoglobin concentration was observed 15 minutes after LVR with PEG-20k and throughout the 24-hour study ($p < 0.01$). Hemoglobin decreased from 9.3 ± 0.7 g/dL at the end of shock to 5.9 ± 0.9 g/dL at LVR-240 ($p < 0.001$). POD1 hemoglobin was 6.5 ± 1.3 g/dL which was not significantly different than the levels measured at LVR-240. Intravascular volume, which similarly decreased in all groups to an average of 51.1% at the end of HS, was restored to 99% ± 4.6% of baseline volume 30 minutes after LVR with PEG-20k. This volume expansion was maintained for 3.5 additional hours. On the other hand, post-LVR intravascular volume peaked 15 minutes after LVR with WB and Hextend to 57.5 ± 5.5% and 66.2 ± 10.5%, respectively. These

increases were not statistically different between the 2 groups but significantly lower than the PEG-20k group ($p \leq 0.001$), (Figures 5 and 6).

DISCUSSION

To our knowledge, this is the first preclinical study to report superior hemodynamic, metabolic, and 24-hour survival outcomes with a non-sanguineous LVR solution compared to whole blood (WB), without definitive resuscitation. All animals resuscitated with PEG-20k after severe class IV hemorrhage survived 24 hours and had a normal NDS, indicating full neurological recovery, while none of the animals in the Hextend group and only 1 out of 6 in the WB group survived more than 4 hours after LVR. Our results strongly suggest that improving OCC after severe hemorrhagic shock with RBC-rich solutions may not be the most crucial target for early resuscitation. Importantly, PEG-20k and WB target two different mechanisms related to HS and resuscitation injury. One is the microcirculatory exchange failure due to cell swelling from the loss of energy-dependent mechanisms responsible for cellular volume regulation (PEG-20k). The second is the OCC depletion directly related to the loss of hemoglobin during hemorrhage. Despite the increasing emphasis on early blood products administration, there is a large body of evidence from observational studies to suggest that prioritizing the normalization of other elements of shock-induced derangements over OCC may be advantageous. Hemostatic resuscitation with a high plasma to RBCs ratio has recently been advocated for posttraumatic HS as data from combat and civilian studies showed potential survival benefits (30-33). A multicenter prospective study of adult trauma patients who received at least one and three units of RBCs in the first 6 and 24 hours after admission, respectively (n = 905), found that higher ratio of plasma and platelet to RBCs was associated with decreased mortality within the first 24 hours. Similar findings were reported in a retrospective analysis of the US military population

serving in Iraq and Afghanistan between 2003 and 2012 and receiving at least one transfusion product within 24 hours of admission (31).

On the other hand, the importance of the microcirculation in hemorrhagic and septic shock has been increasingly recognized with more evidence showing startling disparities between macro- and microcirculatory measures, especially in the early hours of shock. This led to the ongoing efforts to develop microcirculation-related indices in order to identify patients at risk of impeding deterioration which may not be recognizable using traditional monitoring methods, especially in the ICU (17, 18, 21, 34-38). In a recent study of 58 patients with HS who received an average of 6 units of packed RBCs at 3 major trauma centers in the UK, Hutchings et al. found that patients who developed multi-organ dysfunctions had a significantly lower perfused vessels density and microcirculatory flow index measured within 12 hours of ICU admission but a similar cardiac index, compared with patients who did not. Therefore, it is not surprising that the perfused vessel density was a more sensitive predictor of multi-organ failure compared to lowest recorded blood pressure and the highest recorded lactate and cardiac index (21).

The robust biologic effects of PEG-20k are due to its unique effects at the level of the microcirculation. The polymer targets ischemia-induced cell-swelling that hinders microcirculatory blood flow but doesn't change OCC in the pre-capillary macrocirculation like whole blood resuscitation does. PEG-20k is an unconventional polymer that unequally partitions in the microcirculation to establish multiple osmotic gradients for water transfer out of laden parenchymal cells into the capillary space. This response is amplified by its high hydrophilic nature. The reversal of the metabolic swelling-induced microcirculatory exchange failure in shock dramatically increase capillary perfusion and oxygen transfer even under low volume and low OCC states with class IV hemorrhage. This is supported by the baseline return of plasma lactate concentrations after PEG-20k

resuscitation without addition of more OCC. Some of this hypothesized perfusion effect is supported by higher capillary perfusion pressures from rapid volume expansion and part is supported by decreased resistance to flow by decompression of the tissue capillary beds as cell swelling is mitigated. Although we did not directly measure capillary flow in these studies, our previous studies demonstrating dramatic increases in capillary flow after PEG-20k resuscitation in severely shocked rodents (18). In the case of OCC-focused resuscitation, RBCs are largely ineffective because the capillaries are obturated by swollen endothelial and parenchymal cells, which prevents oxygen transfer. Therefore, these studies have demonstrated that it is more important to restore capillary perfusion than add more OCC in severe hemorrhage because doing the former restores oxygen delivery to the microcirculation and pays back oxygen debt in the local tissues, even in the face of a 50% drop in oxygen carrying capacity after hemorrhage. The corollary is that adding OCC (blood RBC products) after shock may be ineffective if the microcirculation is swollen shut by metabolic swelling from ischemia. The ideal world is to both replace OCC after shock, which is now the gold standard, and decompress the microcirculation by impermeant-induced passive water transfer to ensure oxygen transfer to the local mitochondria.

The ability of low volume crystalloid resuscitation with 10% PEG-20k to restore and maintain adequate oxygen delivery to tissues sufficient to result in 24-hour survival without further restoration of oxygen carrying capacity suggest this solution could serve as a partial blood substitute. Administration of this solution in shocked patients may make sense in austere pre-hospital environments where blood products are unavailable. Perhaps these solutions are also useful in hospital settings either in conditions of low blood supply (mass casualty) or simply to effect oxygen delivery of traditional oxygen carriers such as whole blood or blood products. In fact, preliminary studies suggest PEG-20k added to whole blood at the time of resuscitation rescues the failure of the blood alone to resuscitate and survive severely shocked swine (unpublished data). While the

crystalloid alone is shown in these studies to support recovery from lethal shock 24 hours after resuscitation without any additional use of oxygen carriers, the limits of this effect remain unknown. However, a 24-hour survival period associated with normalization of metabolic and central cardiovascular outcomes after resuscitation from lethal shock would be an enormous advancement in pre-hospital care and medical transport.

PEG-20k is rapidly cleared by the kidneys resulting in a two-hour half-life (23, 39). Therefore, the sustained hemodynamic and metabolic effects observed at 24-hours demonstrates the paramount importance of early reversal or prevention of cellular swelling and local microcirculatory failure in achieving effective resuscitation outcomes in severe HS. It seems likely that PEG-20k, through its unique abilities to restore capillary blood flow and oxygen transport after shock, interrupts a vicious cycle responsible for severe and lethal resuscitation injury. Specifically, early restoration of capillary blood flow by normalizing cell volume, restores oxygen transfer to local mitochondria sufficient to not only meet local bioenergetics requirements of the local tissue, but also sufficient to repay the accumulated oxygen debt. With presumably normalized cell ATP levels to fuel energy dependent cell volume control, the tissues maintain capillary flow after shock, even in the face of a 50% deficit in total oxygen carrying capacity. For the first time, these data have unmasked the incredible reserve in oxygen delivery that exists in the system by simply increasing the oxygen transfer efficiency of the microcirculation. If the capillaries can be kept open for exchange, oxygen delivery is adequate even when the OCC is cut by half after severe blood loss. On the other hand, the limited effectiveness with WB and Hextend resuscitation is due to the inability of these components to reproduce the PEG-20k related effect of ‘fluid repatriation’, which allows utilization of water trapped inside the cells to adequately expand the intravascular space and decrease capillary resistance to flow. The dissatisfactory volume expansion and persistent microcirculatory collapse with WB and

Hextend may explain their failure to achieve the same outcomes observed with PEG-20k in our model.

Lastly, the known coagulopathic effects of other polymers like Hextend (16) and the effective volume expansion effects of PEG-20k to produce a dilutional coagulopathy has necessitated a comprehensive analysis of the effects of PEG-20k on coagulation and platelet function. Previous ex-vivo analysis of PEG-20k on coagulation and platelet function of blood from healthy volunteers and from severely injured trauma patients during early admission (40, 41) and in vivo analysis in the same porcine model of HS (39) showed only a mild and reversible coagulopathy in the first 2 hours after IV resuscitation. The mechanisms involve a nonspecific functional thrombasthenia and minor effect on fXIII-induced fibrin cross linking (39-41).

The main limitation of this study is related to the experimental design using a controlled hemorrhage model. Although the controlled hemorrhage nature of our model allows accurate scientific comparisons of the mechanistic targets of treatment, there are limitations in clinical translatability when bleeding cannot be controlled at resuscitation. We also did not evaluate other blood components or different plasma to platelet to RBC ratios that are currently utilized in clinical practice. Instead, we chose to use two control solutions that are used in prehospital resuscitation in the military (Hextend) and in the civilian field (WB, where available). Finally, we did not directly measure capillary blood flow in the pig model but extrapolate our discussions from our data in rodent models where we did.

In conclusion, low-volume resuscitation with PEG-20k based crystalloid solutions resulted in 100% survival for 24 hours in a lethal hemorrhagic shock model, compared to either the same volume of Hextend or whole blood where most animals survived for about 2 hours. Resuscitation with PEG-20k was associated with return to baseline of plasma lactate, arterial blood pressure, and plasma

volume. Survival with PEG-20k was always associated with normal neurological function 24 hours after recovery. This novel crystalloid resuscitation solution should be a superior solution for prehospital use in shocked patients and may have use as a partial blood substitute and as an adjunct to greatly improve resuscitation outcomes with blood and blood products. Finally, these data show that whole blood is not the gold standard for resuscitation and demonstrate that a major mechanism of resuscitation injury in shock is loss of capillary oxygen transfer due to metabolic cell swelling. The use of blood or other oxygen carriers in shock without first addressing this mechanism is futile because the goal is to increase delivery of oxygen to the mitochondria and this requires both oxygen carrying capacity and efficient transit through the microcirculation to be effective.

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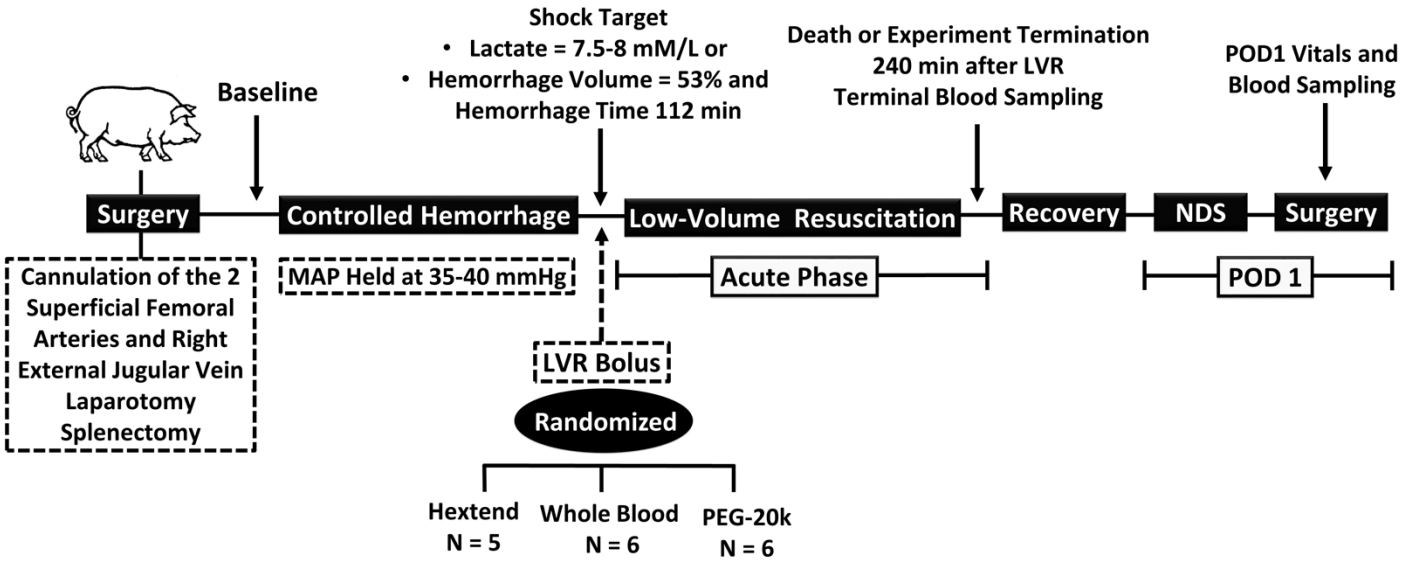
Table 1: Baseline information and 24 hours survival rates and neurologic deficit scores after low volume resuscitation

	Hextend N=5	Whole blood N=6	PEG-20k N=6	p value*
Weight (kg)	36.6 ± 4.2	34 ± 2.1	34 ± 2.6	NS
Baseline MAP (mmHg)	84.8 ± 9.6	86.2 ± 11	87.2 ± 14.6	NS
Baseline Lactate (mmol/L)	2.3 ± 1.1	1.8 ± 0.5	2.2 ± 0.7	NS
Baseline Hematocrit (%)	28.9 ± 1.2	28.9 ± 2.1	29.8 ± 1.6	NS
Baseline Alanine transaminase (u/L)				
Baseline Creatinine (mg/dL)	1.2 ± 0.2	1.1 ± 0.1	1.2 ± 0.2	NS
LVR Time (minutes)	69 ± 73.9	92.5 ± 104.5	240 ± 0	0.003
24-Hour Survival Rate N (%)	0 (0)	1 (16.7)	6 (100)	0.001
Survival Time (hours)	2.2 ± 1.4	5.2 ± 9.2	24 ± 0	< 0.001
24-Hour Neurologic deficit score		40/400	0/400	
Overall performance category (OPC):				
OPC 1: normal	0 (0)	0 (0)	6 (100)	
OPC 2: moderate disability	0 (0)	1 (16.7)	0 (0)	
OPC 1: severe disability	0 (0)	0 (0)	0 (0)	
OPC 1: Coma	0 (0)	0 (0)	0 (0)	
OPC 1: brain death or death	5 (100)	5 (83.3)	0 (0)	

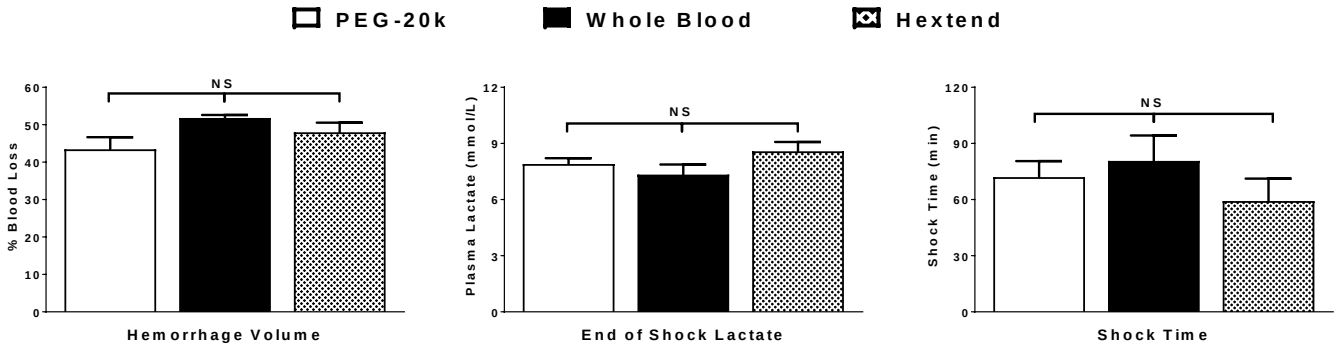
*P values are for the 3 group comparison (one-way ANOVA)
PEG-20k: polyethylene glycol 20,000 Daltons; MAP: mean arterial pressure; NS: not significant

Figure 1. The experimental design of this study



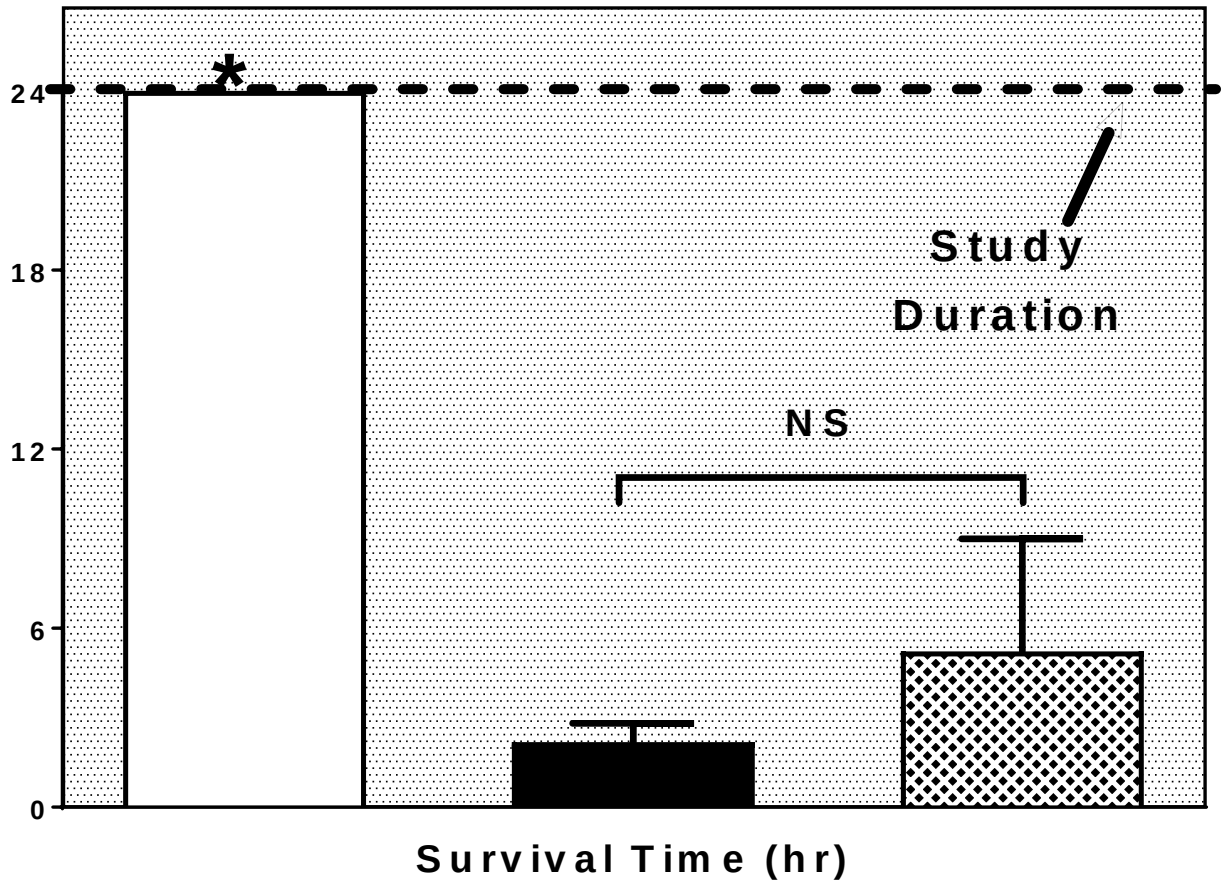
LVR: low-volume resuscitation; MAP: mean arterial pressure; PEG-20k: polyethylene glycol 20,000 Daltons; NDS: neurologic assessment score; POD1: postoperative day 1

Figure 2. Measures of shock reached at the end of the hemorrhagic shock period, before the start of low-volume resuscitation



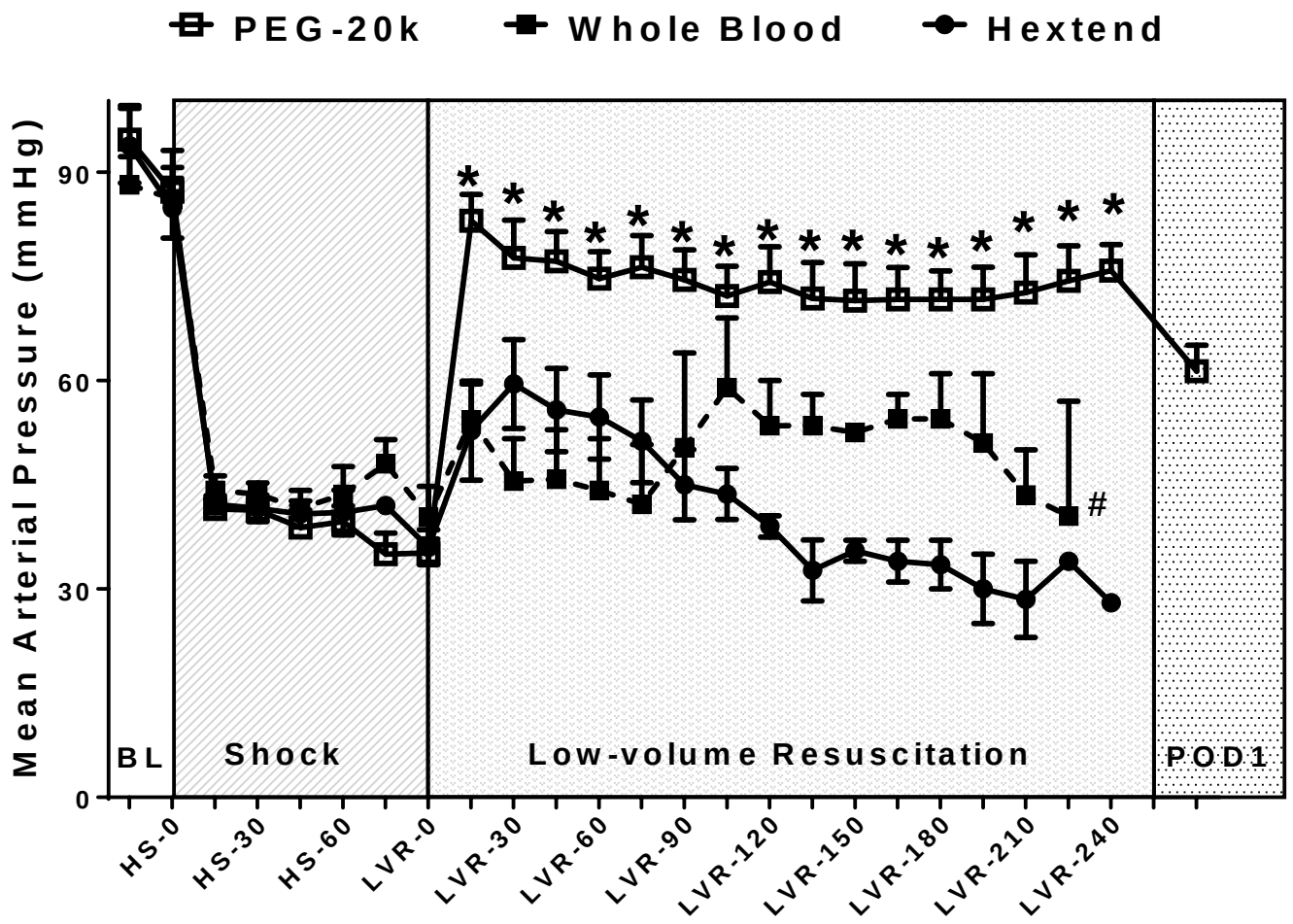
PEG-20k: polyethylene glycol 20,000 Daltons; NS: not significant

Figure 3. Survival time after low-volume resuscitation



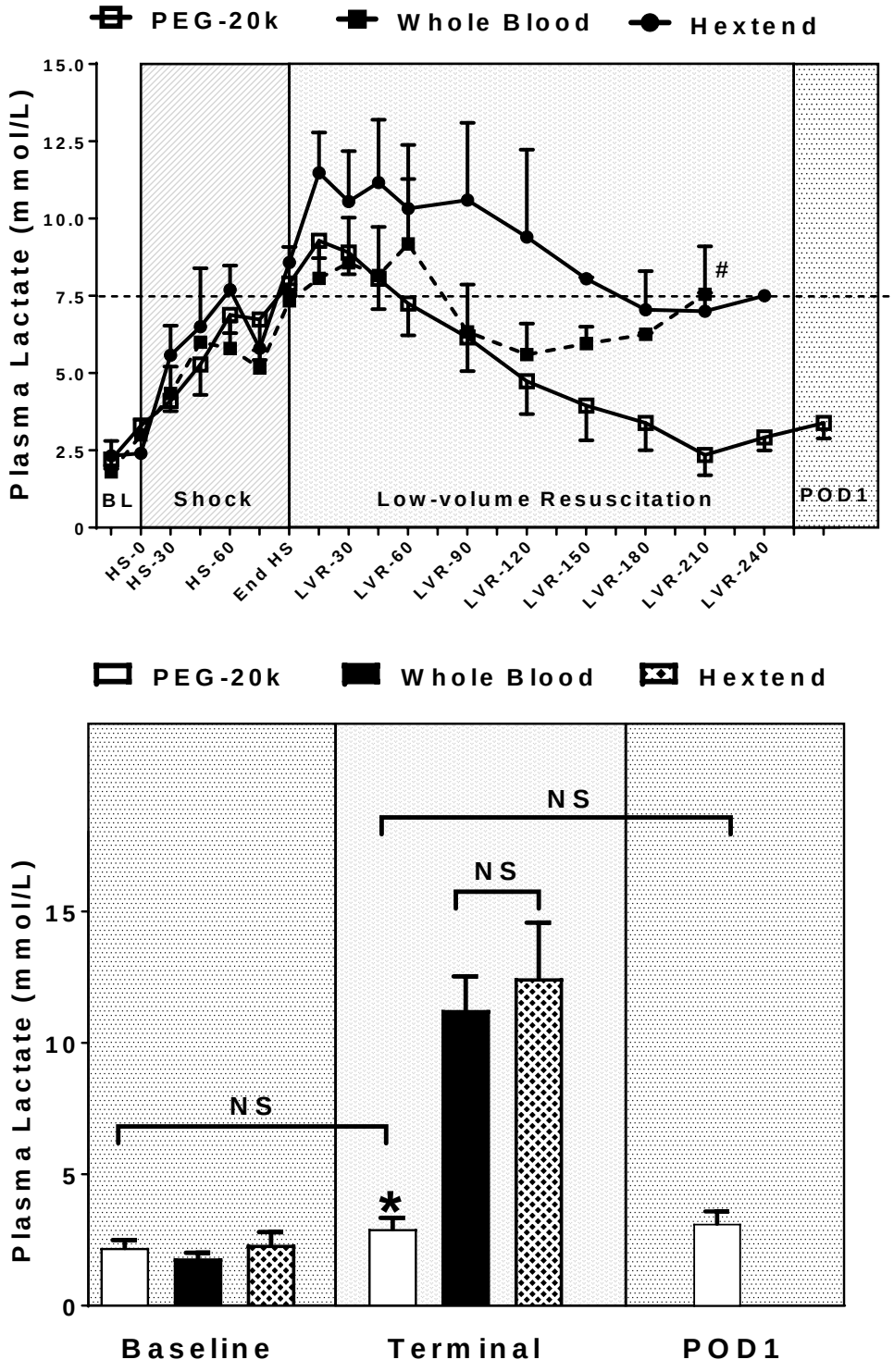
LVR: low-volume resuscitation; PEG-20k: polyethylene glycol 20,000 Daltons; NS: not significant

Figure 4. Changes in mean arterial pressure during hemorrhagic shock, low-volume resuscitation and on postoperative day 1



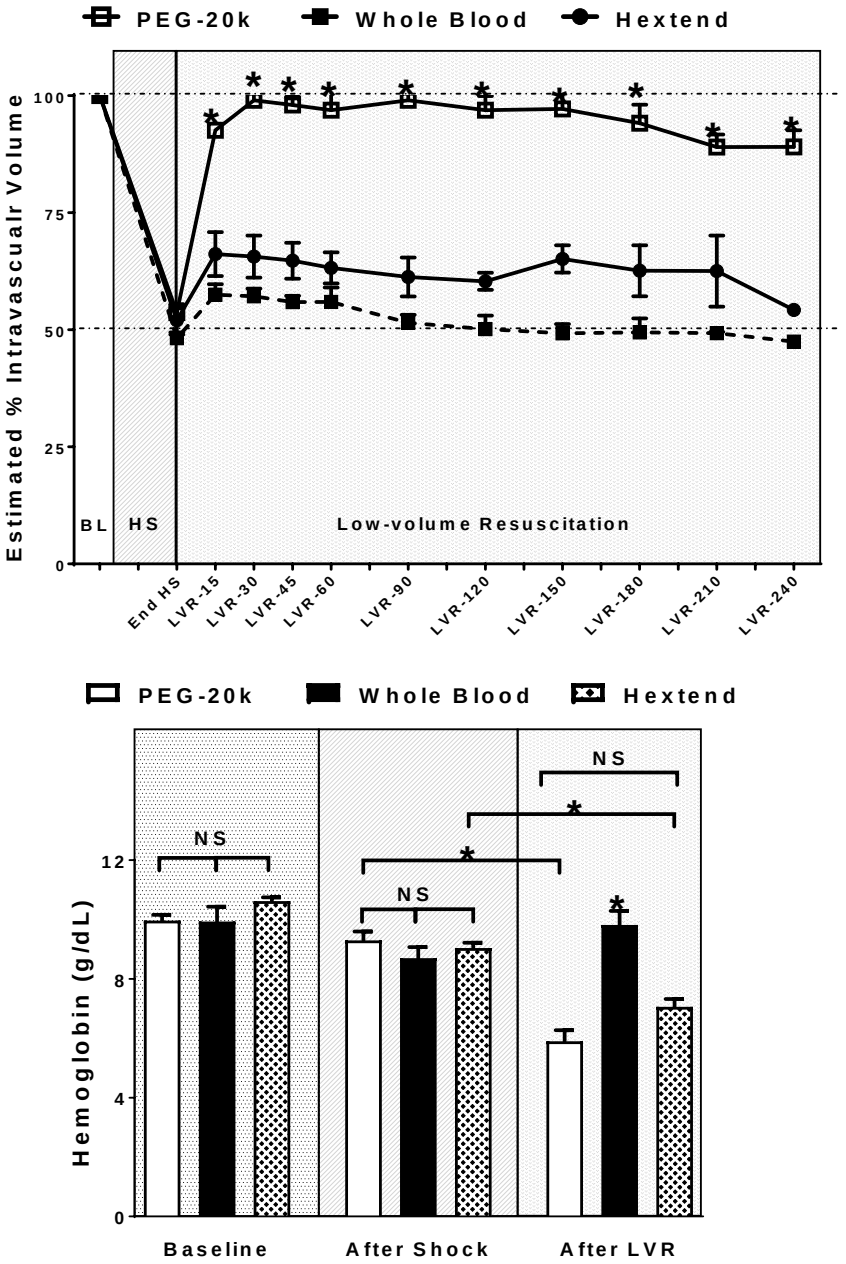
LVR: low-volume resuscitation; PEG-20k: polyethylene glycol 20,000 Daltons; BL: baseline; POD1: postoperative day 1

Figure 5. Changes in plasma lactate during hemorrhagic shock, low-volume resuscitation and on postoperative day 1



LVR: low-volume resuscitation; PEG-20k: polyethylene glycol 20,000 Daltons; BL: baseline; POD1: postoperative day 1

Figure 6. Changes in estimated intravascular volume during the 4 hours early post-resuscitation (acute) phase of the experiment and hemoglobin levels during hemorrhagic shock, low-volume resuscitation and on postoperative day 1



LVR: low-volume resuscitaton; PEG-20k: polyethylene glycol 20,000 Daltons; POD1: postoperative day 1; NS: not significant