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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. Specifically, PEG-20k (MW 20,000) produced optimal resuscitation outcomes compared to smaller or larger polymer sizes. 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, probably by surface binding, but is clinically moot at currently used concentrations in shock.

15. SUBJECT TERMS

Crystalloid IV fluids, hemorrhagic shock, osmotic effects, oxygen debt repayment, tissue swelling

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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 begin exploring 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

3. **ACCOMPLISHMENTS:**

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

I. To determine the effects of PEG-20k LVR solutions in a rodent survival model and to explore the pharmacokinetic behavior of PEG-20k in vivo under shock conditions.

II. To determine the molecular mechanisms of action of PEG-20k solutions on the dose dependent hypocoagulative state seen in TEG assayas of clotting whole human blood.

III. To assess PEG-20k based LVR solutions on coagulation and platelet function in a pre-clinical swine model of lethal hemorrhagic shock and to determine peak blood levels and half-life of elimination of PEG under these conditions, as modeled for use in clinical resuscitation.

IV. To assess the effects of PEG-20k LVR solutions in recovery and survival in swine after lethal hemorrhage.

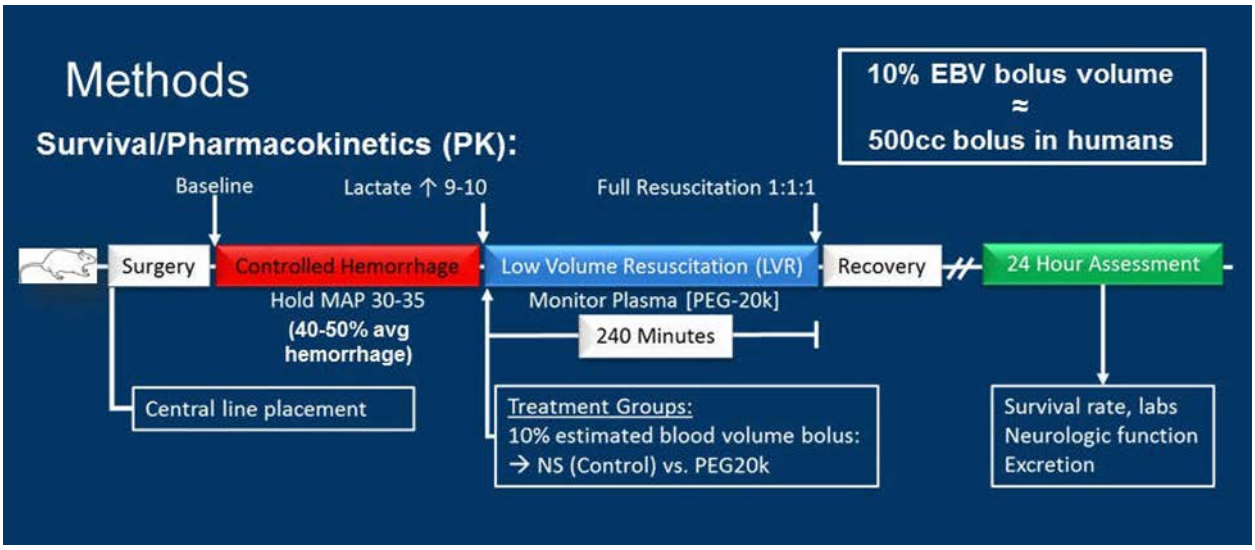
V. To determine how PEG-20k LVR solutions perform in a rodent model of UNCONTROLLED hemorrhagic shock.

What was accomplished under these goals? Six projects are described that highlight our accomplishments over the last year.

Effects of PEG-20k low volume resuscitation in a survival model of lethal hemorrhagic shock in the rodent model.

The effect of PEG-20k LVR in acute shock studies is well known. These solutions increase tolerance to the low volume state 5-20 fold, depending on the exact model, and do so by dramatically increasing the efficiency of microcvascular oxygen transfer to tissues secondary to the non-energetic movement of metabolic cell and tissue

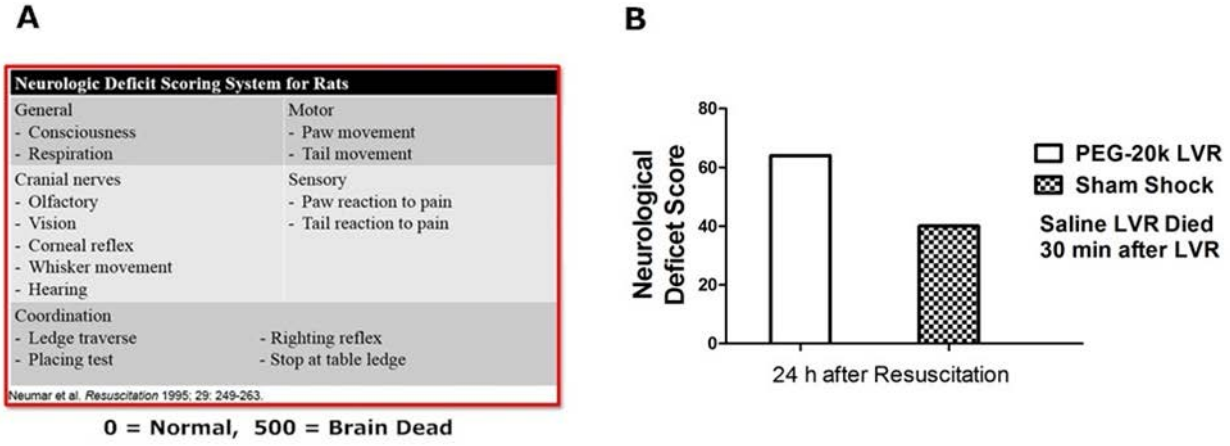
water out of the tissues and into the capillaries. However, survival behavior in this model is not known and was studied. A cartoon illustrating the model is shown in Figure 1.



In brief, anesthetized adult rats were catheterized and hemorrhaged to an oxygen debt characterized by a plasma lactate of 9-10 mM, given a low volume resuscitation of 10% PEG-20k in LR at a volume equal to 10% of the estimated blood volume, given full resuscitation 240 min after LVR with 10% blood volume dose of autologous whole blood adjusted to a Hct of 50% (to emulate clinical 1:1:1 solutions), and finally recovered for 24 hours. Survival and outcomes were measured the next day. Since all of the saline volume controls die 30-60 minutes after LVR, there was no direct comparator group 24 hours after resuscitation (because they all died). One important outcome measured the next day, besides survival and cardiovascular function, was neurological and brain function as assessed by a neurological deficit scoring test.

Neurological deficit scoring was measured in a previously used and published testing protocol described in Figure 2.

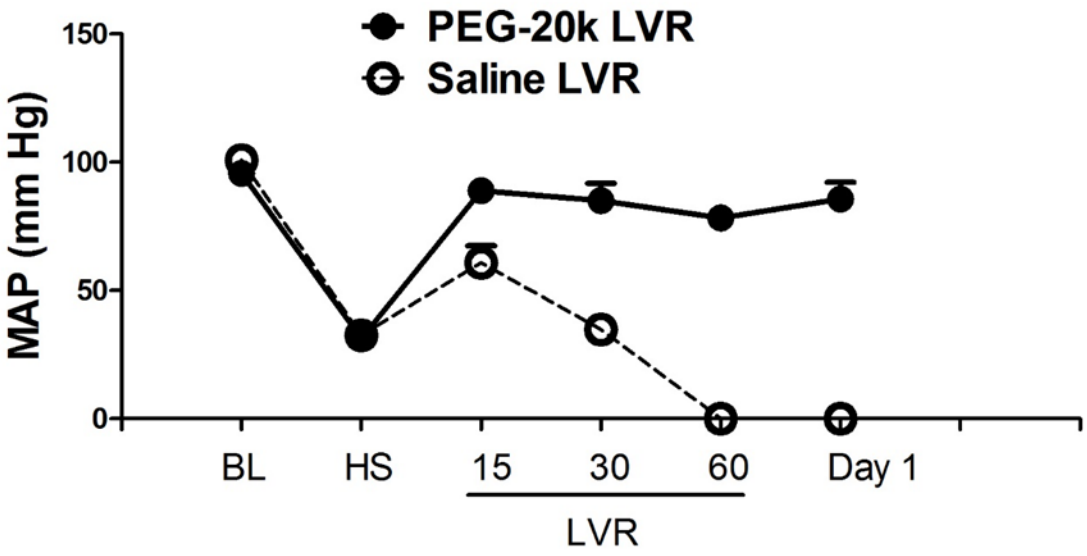
Figure 2



In this test, rats that recover from shock, resuscitation, surgery, and anesthesia the following day are given a battery of neurobehavioural testing (conducted by experts at VCU not directly associated with our studies) that consider general behaviour, cranial nerve function, moror function, and coordination (Fig. 2, Panel A). The score range is from 0, which is normal to 500, which indicates rats with clinical brain death. In our shock study, the saline volume controls all died before they could be tested but the PEG-20k resuscitated rats that all survived scored 62, which indicated some deficits in vision and hindlimb coordination. However, a sham operated group of rats treated identically, except without hemorrhage and resuscitation, scored 42 and also had blindness and some hind limb paralysis. It was determined that the surgery, anesthesia, and vascular cut-down of the hind limbs produced blindness and some paralysis in surviving rats. These effects are NOT attributable to the PEG-20k resuscitation or shock. The blindness may likely be due to persistent corneal dryness associated with prolonges gas anesthesia and breathing of dry oxygen mixtures passing over the eyes for 6 hours. Hind limb deficits result from vascular cut down of the hind limbs during the study, which causes coordination defects during next day testing. The point is that the surviving rats had essentially normal neurological function after recovery and the defects that were observed were technical and not related to the shock or the resuscitation.

While none of the saline LVR controls survived more than 1 hour after resuscitation, 100% of the PEG-20k resuscitated rats survived not only the 240 minute LVR period but also the 24 hour recovery period. The mean arterial blood pressure in those surviving rats are compared to sham operated rats and is shown in Figure 3

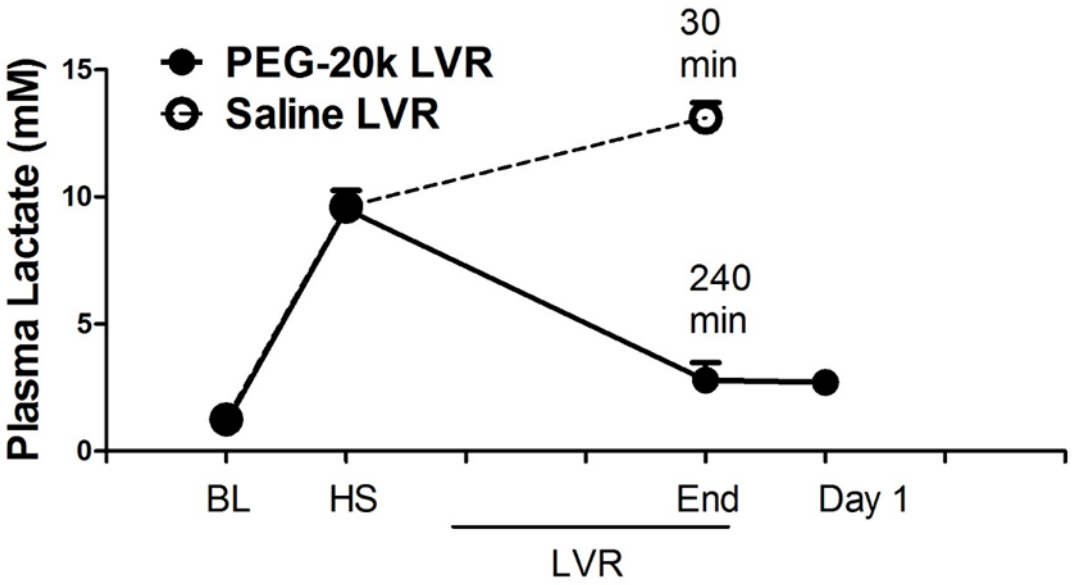
Figure 3



The rats resuscitated with PEG-20k low volume resuscitation crystalloids were able to maintain their blood pressure, not only for the 240 min LVR period, but also over the next 24 hours after full resuscitation with whole blood and after recovery the next day from anesthesia. The volume controls resuscitated with the same volume of saline (LR is used mow since it will be part of the pivitol solution for FDA testing) all died within an hour of LVR and did not survive to the next day testing period.

Consistent with survival and blood pressure results are the metabolic effects observed in PEG-20k resuscitated rodents. These data are shown in Figure 4.

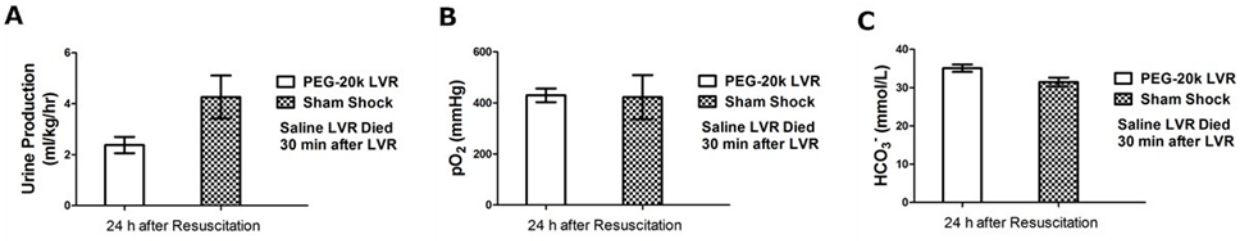
Figure 4



In both groups, lactate after hemorrhage rose to the same value (10 mM) because of the experimental design but differed thereafter. In the saline resuscitation controls, lactate continued to rise but fell almost to baseline in the PEG-20k resuscitated group. The LVR end-time was dramatically different too. Target lactate (10 mM) after LVR was hit in 15-30 minutes in the saline group because the saline failed to restore tissue perfusion but the target was not reached, even after 240 min, in the PEG-20k LVR group. We don't know what the true LVR time (a measure of tolerance to the low volume state) is in the PEG-20k group because we arbitrarily cut off the study after 240 minutes so we could administer full resuscitation and recover the animals. Finally, the PEG-20k resuscitated rats all survived the next day and demonstrated almost normal plasma lactate values, indicating a restoration and maintenance of tissue perfusion

Other outcome variables in surviving rats are shown in Figure 5 (but not in saline resuscitated rats because they did not survive the recovery period).

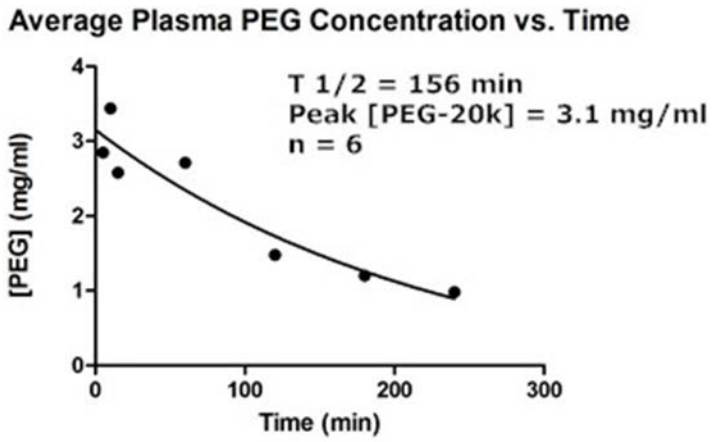
Figure 5



Urine output was not different in rats that were resuscitated with PEG-20k LVR solutions compared to sham operated rats (panel A) indicating return to normal renal function. Similar changes were seen for pO2 (panel B), and bicarbonate (panel C), indicating a return to normal pulmonary blood flow, gas exchange, renal function, liver function, and metabolism with PEG-20k resuscitated rats after lethal shock.

A final objective of this quarter was to begin understanding the pharmacokinetics of PEG-20k after administration to shocked rats. The resuscitation strategy for both experimental studies and for clinical field applications is to administer crystalloid solutions to shocked patients at a volume of no more than 10% of the estimated blood volume, which represents about 500 ml in an adult with a blood volume of 5 liters. This theoretical 1:9 dilution of the 100 mg/ml PEG-20k solution should result in a peak plasma concentration of PEG-20k of 10 mg/ml. This dilution was used to test the coagulation and platelet function behavior in ex-vivo whole blood testing in human volunteers and trauma patients. In order to extrapolate those results with actual resuscitation environments, we need to understand what the peak blood levels and circulating half-life of this solution actually is. To that end, we included a FITC-labeled PEG-20k probe in the PEG-20k LVR solutions used to resuscitate the rats in this study. This allowed us to sample plasma over time and monitor PEG-20k blood concentrations using simple fluorometric analysis of the plasma. The average results of 6 of these studies is shown in Figure 6.

Figure 6

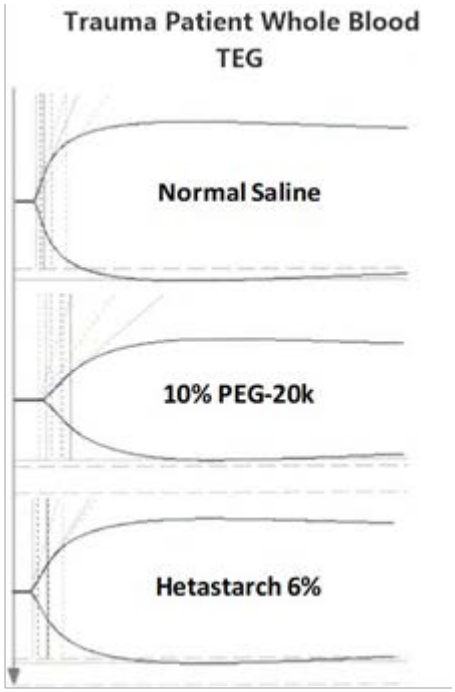


Eight kinetic studies were conducted yielding 6 that yielded technically usable data for analysis. The results surprisingly show a peak plasma concentration of only 3 mg/ml, which is about 3 fold lower than what would be expected from the calculated dilution, based on estimated blood volumes. Furthermore, the circulating half-life of elimination was found to be only about 2 ½ hours. Practically, this means that the concentration of PEG-20k is well below the 10 mg/ml that was used in the human blood TEG testing that demonstrated a significant hypocoagulative state. This is obviously very good news because this suggests we can achieve all of the cardiovascular and metabolic benefits of PEG-20k resuscitation but avoid the coagulopathies that can occur at higher concentrations. Knowing this behavior also shows us doses that can be used by these solutions to deliberately cause a state of reversible platelet passivation, such as during cardiopulmonary bypass or in trauma patients with severe orthopedic trauma that are at high risk of developing DVT or PE complications. Finally, we also determined the predominant route of excretion of the fluorescently labeled PEG-20k tracer was via renal excretion into the urine with a small percentage excretion into the feces, probably by biliary clearance (data not shown).

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 7). 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 7



Since the angle and k values were altered with PEG, explored if fibrinogen and von Willebrand factor concentrations were altered. As seen in figure 8, 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 8

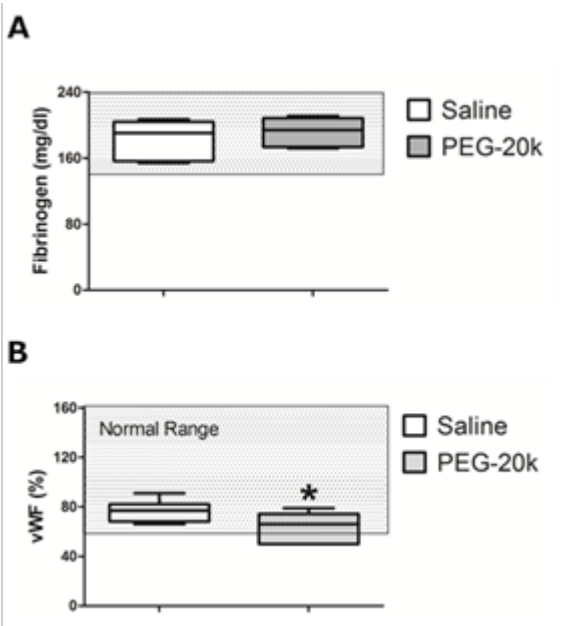
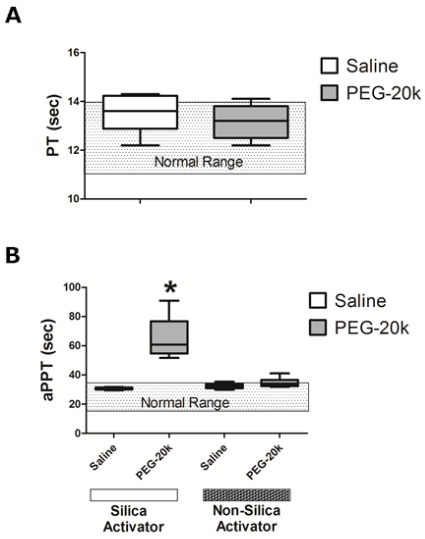
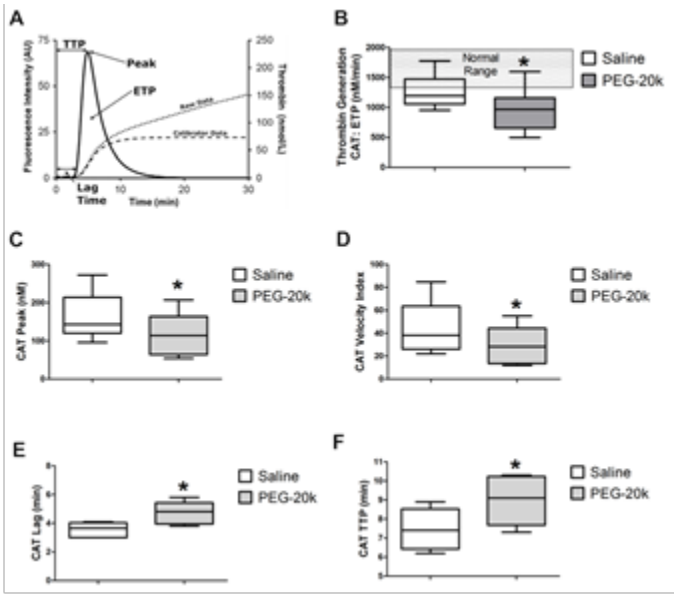


Figure 9



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 9, 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 10

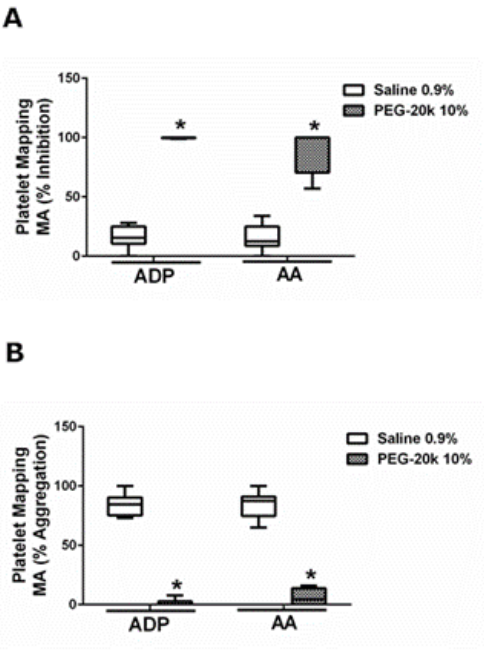


Thrombin generation by the system, from both platelet and chemical plasma components, is shown in Figure 10 for both PEG-20k and saline diluted platelet rich plasma (PRP) using low dose (1 pM) tissue factor as the trigger. We detected a very slight but significant decrease in most of the outcomes of 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 11. 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 PSelectin that are detected by specific binding of antibodies to both (PAC1 and anti-CD62P, respectively). These data are shown in Figure 12 for both saline and PEG-20k diluted PRP samples.

Figure 11



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 12

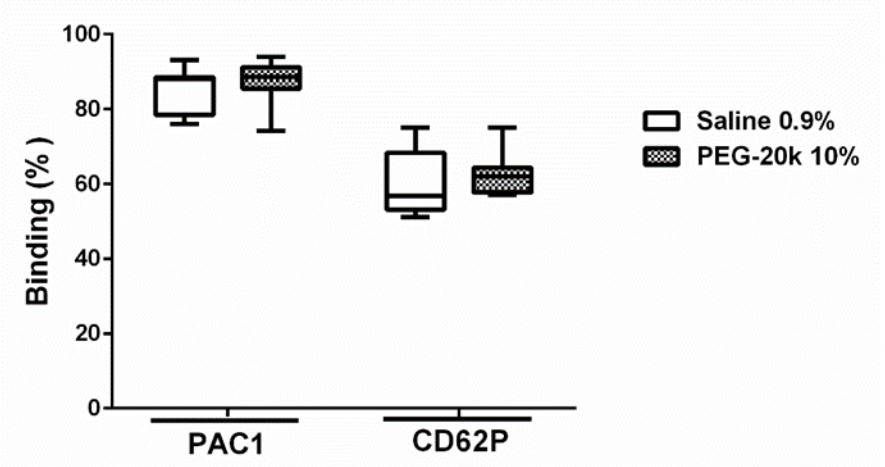
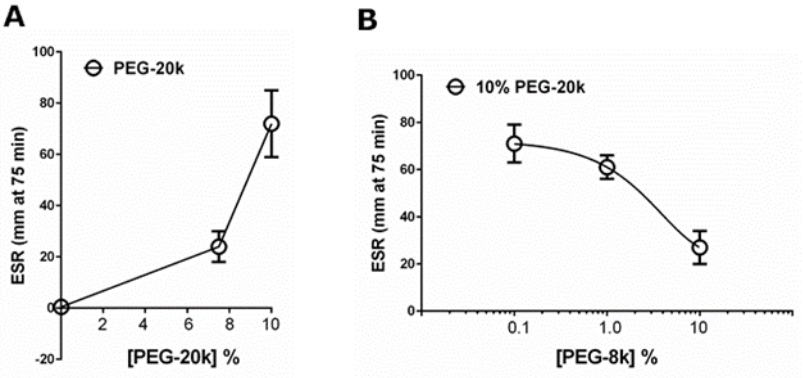


Figure 13

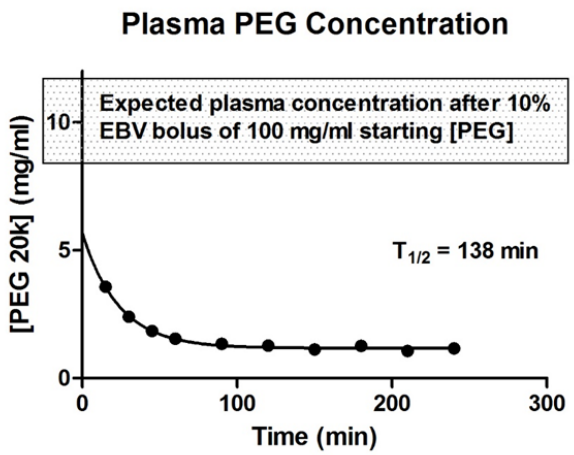


If this binding were to occur in platelets too, then some platelets may be functionally removed from binding with fibrin, fibrinogen, and adhesion molecules to alter the platelet component of clot formation, as documented clearly in these two studies. This proposed parallelism between PEG-20k interactions 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 13) 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 14



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 14.

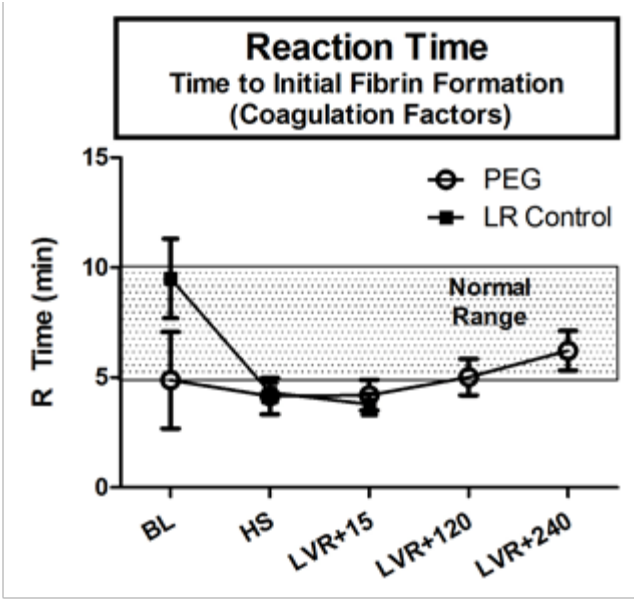
These data clearly show that the peak PEG-20k concentrations in the blood are at about 5 mg/ml, which is far lower than the 10% needed to cause coagulopathies. Therefore, the previously observed hypocoagulation seen after PEG-20k dilutions in whole human blood are dose dependent and likely will

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.

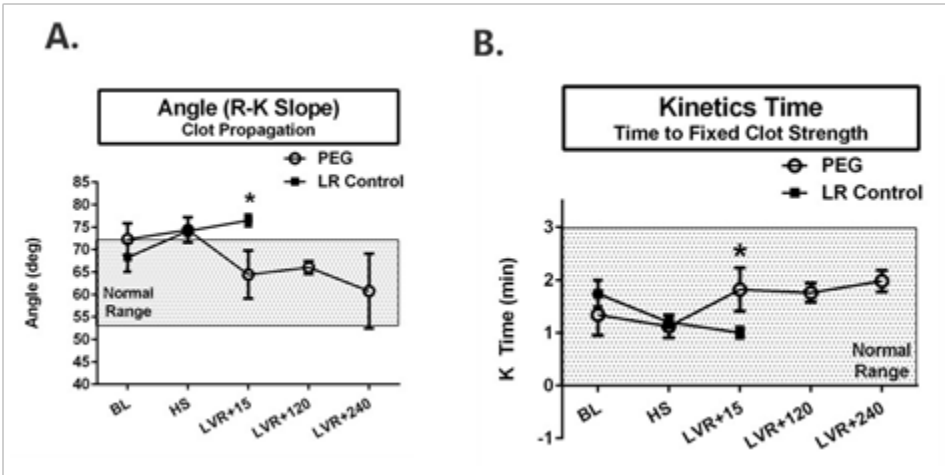
Methods and Model: Pigs (40kg) were anesthetized and shocked by bleeding the animals to an arterial pressure of 35 mm Hg until their oxygen debt achieved a set value, which was standardized to one of the three endpoints (determined empirically from the previous acute studies):
1. Plasma lactate reaches 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 15



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 15 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.

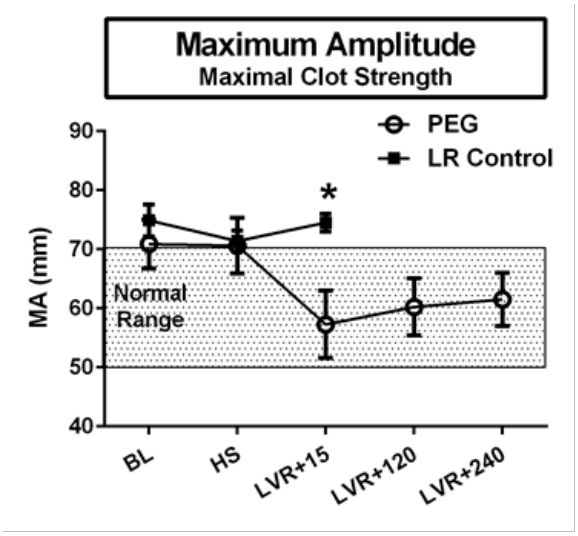
Figure 16



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 16, 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 17).

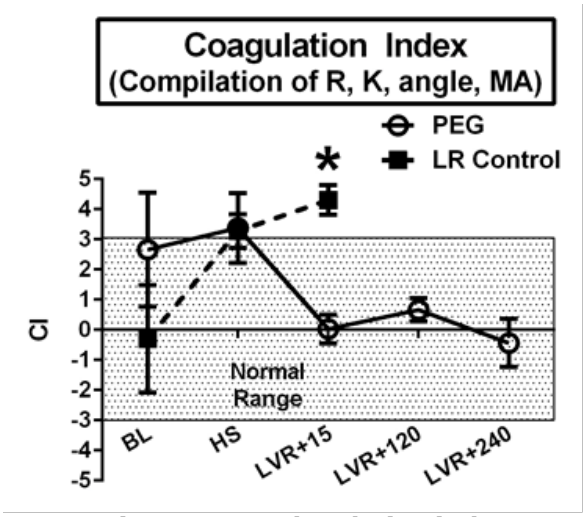
Figure 17



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 18. The CI is a mathematical modeling of multiple TEG attributes including R, k, angle, and MA.

Figure 18



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 overall survival in shocked pigs.

An important objective for this year was to begin 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. To date we have done 3 pigs treated with PEG-20k LVR solution ands survived the next day. We have not done any LR treated pigs because none of them survive past 30 min after LVR. We will, however, begin randomizing PEG-20k treated pigs with those treated with an equal volume of Hextend. The methods for the survival pig shock protocol are the same as the acute studies described earlier. A summary of the effects on survival is shown in Tables 1 and 2 where we compared baseline values to values obtained 2 days after recovery from a lethal hemorrhage, a recovery only possible using PEG-20k solutions.

Table 1: Lab values at baseline and 2 days after severe shock, 4 hrs PEG-20k LVR, and Full Resuscitation

	Lactate	Hb	ALT	Cr	Amylase	K	PLT	WBC
Baseline								
X	2.2	8.6	9	0.95	1110	4.4	219	17.4
SD	1.7	1.1	1.8	0.07	147	0.1	40	4.8
POD-2								
X	1.1	6.0	82	1.0	1227	5.6	246	14.7
SD	0.7	0.6	5.7	0.4	229	0.5	95	0.8

The lab values for these pigs (Table 1) shows they are normal except for a mild elevation of liver transaminase values (ALT). For cardiovascular function and coagulation (Table 2), we see that the

survival pigs are normal. The mean arterial pressures on day 2 post-recovery are a bit lower than baseline but the pigs have not had any further resuscitation of voilume, other than what water they drink. The TEG values after recovery are normal too and suggests that worries about post-resuscitation coagulopathies or renal failure are not founded. The lesson is that PEG-20k is highly effective in recovery without significant side effects, including those commonly seen with hextend.

Table 2: Hemodynamic and TEG values at baseline and 2 days after severe shock, 4 hrs PEG-20k LVR, and Full Resuscitation

	MAP	HR	Temp		R	Angle	MA	CI
Baseline								
X	102	92	37.9		5.33	65.5	74.3	2.2
SD	27	8.3	0.3		0.68	10.1	7.3	1.3
POD-2								
X	68.0	81	36.9		4.95	72.4	82.9	4.0
SD	4.6	9.8	1.3		0.64	5.4	1.9	0.9

Finally, a neurological deficit score was assigned to each pig that recovered after shock to determine brain neurological status and function. An overall score of 1 was normal and a score of 5 represented brain death. All three pigs in this group (receiving PEG-20k LVR) recovered fully (100% survival) and had an overall neurological deficit score of 1 (normal).

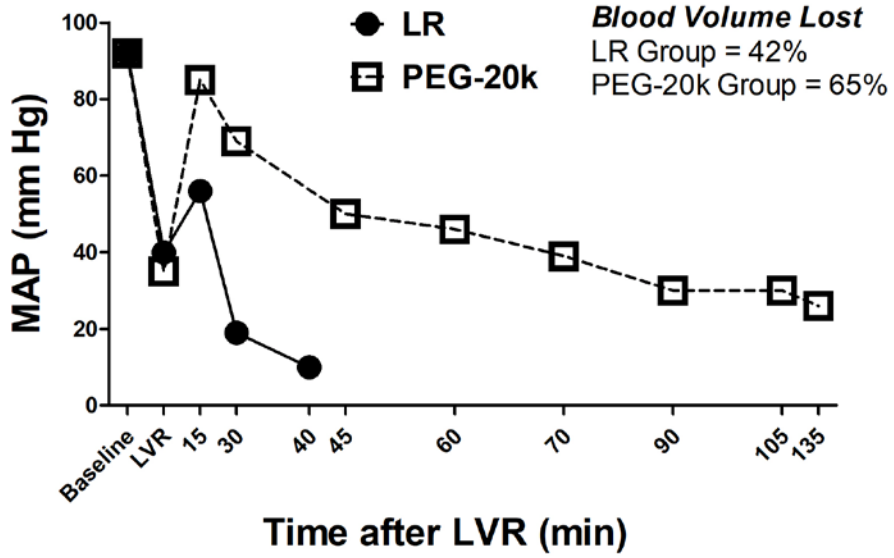
Uncontrolled hemorrhagic shock in rodents

An important objective for the following year is 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 have started that project and report both the model and the first 3 studies, which provide a small insight as to the trajectory of the data for next year.

The model in rats uses two sources of bleeding, a laceration of the spleen and a severed tail, both of which result in a mixted venous and arterial blood loss consistent with traumatic injuries. Furthermore, surgical wounding also plays a role in the cardiovascular and metabolic responses to hypovolemia. Blood is collected from the tail bleed by direct collection into a tube while blood loss from the spleen laceration is also collected into a pre-weighed vial and pre-weight gauze pads. As the uncontrolled bleeding progresses, arterial blood is sampled over time to assess blood gases, metabolites, and lactate. The arterial blood pressure is continuously monitored and not allowed to drop below 35 mmHg. When the plasma lactate levels reach 7-8 mM, the low volume resuscitation (LR or PEG-20k) is administered intravenously at a volume equal to 10% of the estimated blood volume, a true low volume resuscitation. Therats are monitored until death and outcomes are continuously recorded. These include arterial blood pressure, heart rate, temperature, arterial blood gases, including lactate. The survival time is also recorded as an outcome.

In this model, with only 2 rats in each group, we observed a striking benefit of the PEG-20k fluid compared to the LR volume control. Figure 19 shows the effects on mean arterial pressure (MAP) over time in both groups.

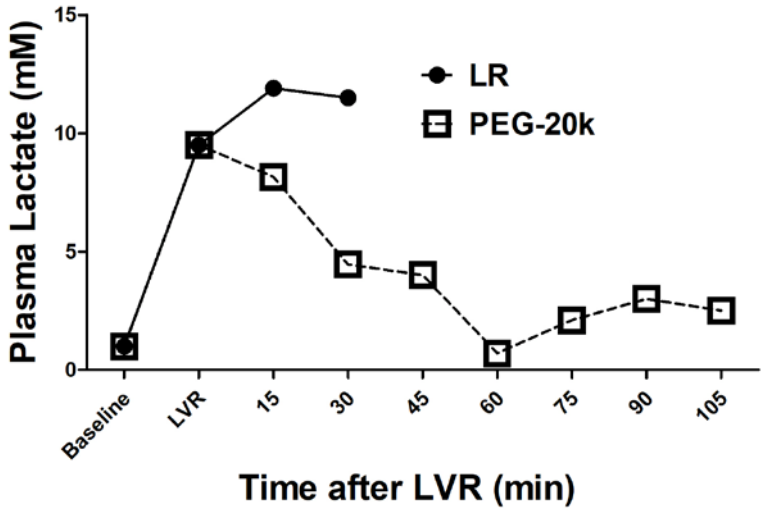
Figure 19



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, despite a much larger hemorrhage volume in the PEG-20k group. The larger amount of bleed volume probably occurred because the pressures were higher, which caused more blood loss.

A similar story is told when the outcome is the plasma lactate concentrations during resuscitation (Figure 20). 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 20



What opportunities for training and professional development has the project provided? The laboratory actively trains surgical residents, non-surgical residents, post-doctoral fellows, graduate students and junior faculty in the school of medicine. These people that participated in the conduct of the studies were given extensive opportunities to learn how to technically conduct shock and laboratory studies, analyze data, design experiments, and present and publish results. In this annual report period, Dr Nina Wickramaratne, Dr Jad Khoraki, and Dr. Loren Liebrecht participated as resident surgeon and post-doctoral trainees. This July, new trainees will move into these positions to continue the work on the project, under the direct supervision of Dr. Mangino and his staff. Furthermore, PhD students (Ria Fyffe), medical students, and countless undergraduate students train in the lab under these projects.

How were the results disseminated to communities of interest?
The results of the last year have been widely disseminated at local VCU seminars and training competitions, at national and international shock and trauma societies, and at the American College of Surgeons meetings. These data are also disseminated by publication in the scientific literature (PLoS1, J. Trauma, and J. Phar. Exp. Ther.).

What do you plan to do during the next reporting period to accomplish the goals?
Similar studies are planned to continue progress on the goals of the project. Shock studies in the rodent and pig model will be used to assess survival after LVR with PEG-20k solutions and compared to clinically relevant alternatives such as LR, Hextend, and blood products (1:1:1). Additional studies on uncontrolled shock will ramp up in rat studies.

IMPACT:
The impact of this project and these results are huge in trauma resuscitation, especially in pre-hospital setting for military and civilian shock. The finding of the safety of the PEG-20k based LVR solutions at effective concentrations has a great impact on moving the commercialization and FDA approval forward in plans for deployment. Since these studies are IND- or IDE-enabling and because we are designing the experiments with FDA approval in mind, we feel we have a significant leg up in this area. Our plans are for a pre-IND meeting with FDA this fall/winter. We have secured an FDA consultant in Norfolk, VA to help with the IDE submission since he believes we can convince FDA that the route to approval is IDE and not IND. A phase-1 dose escalation trial has already been designed for use in normal blood donors. The impact of these data to date on our IND application are very significant.

What was the impact on the development of the principal discipline(s) of the project?
We believe that these data reorient our thinking about the major mechanisms of tissue reperfusion injury. The old ideas of free radical injury and inflammation should give rise to the major importance of metabolic cell and tissue swelling and the “no reflow” phenomenon because of the huge biological effect that we repeatedly see with specifically sized PEG polymers in shock and local I/R injury models. This reorientation in thinking about the major mechanism of reperfusion injury is strengthened by our “locking down” of the mechanisms of how this material works in our shock models. Our experiments to date have incorporated mechanistic studies that clearly suggest the effects are due to the non-energy dependent movement of intracellular water out of tissues and into capillary spaces in order to decompress the local microcirculation and allow for extremely efficient oxygen transfer in the microcirculation under very low volume states as seen in severe hypovolemic shock. This reorientation of thinking also helps us design smarter solutions in the future and in customized tissue beds like CNS to mitigate reperfusion injury in spinal cord injury and TBI

What was the impact on other disciplines?
As discussed above, the implications for reperfusion injury in any other aspect of medicine are applicable to this work since they may all share similar mechanisms of tissue reperfusion injury. Clearly, these developments will be used to treat pre-hospital shock but a myriad of other conditions that all involve derangements in volume control secondary to tissue ischemia. Such applications being considered by

VCU include abdominal and extremity compartment syndrome, neuro-trauma such as spinal cord injury and TBI, volume control in cardiopulmonary bypass and ECMO, CPR, prevention of critical illness in the ICU, ED resuscitation, septic shock, burn resuscitation, pre-hospital trauma resuscitation in civilian and military transports, peripheral vascular disease and chronic heart failure, and space medicine.

What as the impact on technology transfer?
Technology transfer is a very important aspect of this work. Every study that reinforces the clinical importance, biological mechanisms, and safety profiles of the new LVR solutions and platform, improves tech transfer for deployment.

What was the impact on society beyond science and technology?
Whenever healthcare can be impacted to this degree, society benefits from a more productive, economical, and fruitful life for its members.

4. **CHANGES/PROBLEMS:**
There are no problems in the project. In the original study proposed to test the dose response of PEG-20k to prevent over pressure during resuscitation is now moot since we discovered that over pressure with PEG-20k only happens in the rodent model. Where the mean arterial pressure would rise to 100 mmHg after resuscitation in the rat, which may cause more bleeding in uncontrolled shock, mean arterial pressures only rose to about 60-65 mmHg in the clinically relevant porcine shock model. Since the pig represents a more real picture for patients, we feel that 60 mmHg is the perfect pressure that allows good local perfusion but limits further bleeding or risk of "pop the clot" effects. Therefore, we feel it is more important to use the saved resources from not conducting those studies to engage in more IND/IDE-enabling studies like longer term survival and acute toxicity studies, or whatever FDA may ask of us following our pre-IDE meeting. .

Changes in approach and reasons for change

See above

Actual or anticipated problems or delays and actions or plans to resolve them

None.

Changes that had a significant impact on expenditures

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

Significant changes in use or care of human subjects

None

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

5. **PRODUCTS:**

None to report, outside of the already technology being developed and commercialized

Publications, conference papers, and presentations.

1. Wickramaratne, N, Kenning, K., Reichstetter, H., Blocher, C., Li, R., Aboutanos M., and **Mangino MJ.** Acute Resuscitation with Polyethylene Glycol-20k: A Thromboelastographic Analysis. J. Trauma, In Press
2. 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. PLoS1. In Press
3. Liebrecht, L., Newton, J., Martin, E., Brophy, D., Wickramaratne, N., Jayaraman, S., Han, J., Aboutanos, M., and **Mangino, M.J**. Mechanistic Analysis of the Coagulation and Platelet Function Profile of Novel Polyethylene Glycol Based Low Volume Resuscitation Solutions. J. Pharm. Exp. Ther. (In Press)
4. J Yang, Y Xiao, EY Quan, Z Hu, Q Guo, C Miao, JL Bradley, MA Peberdy, **M**. **Mangino**, J. Ornato, and W. Tang. Effects of Polyethylene Glycol-20k on Postresuscitation Myocardial and Cerebral Function in a Rat Model of Cardiopulmonary Resuscitation.
   Critical care medicine (In Press)
5. **MJ Mangino**, LK Liebrecht, EJ Martin, N Wickramaratne, K Kenning, MECHANISMS OF NOVEL POLYETHYLENE GLYCOL BASED LOW VOLUME RESUSCITATION SOLUTIONS ON COAGULATION AND PLATELET FUNCTION
   SHOCK 49 (6), 47-47, 2018
6. N Wickramaratne, L Liebrecht, H Reichstetter, R Fyffe-Freil, C Blocher, and **M.J. Mangino.** Effects of Polyethylene Glycol 20,000 on the Management of Brain Dead Organ Donors in a Canine Model
   AMERICAN JOURNAL OF TRANSPLANTATION 18, 29-29, 2018
7. J Yang, C Miao, Y Xiao, W Huang, Z Hu, Q Guo, X Wu, J Bradley, M. Peberty, M. **Mangino, J**. Ornato, and W. Tang. Effects of Polyethylene Glycol-20K on Post-Resuscitation Survival and Neurological Function in a Rat Model of Cardiopulmonary Resuscitation
   Circulation 136 (Suppl 1), A16699-A16699

Books or other non-periodical, one-time publications.

None

Website(s) or other Internet site(s)

None

Technologies or techniques.

None

Inventions, patent applications, and/or licenses

None

Other Products

None

6. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

No Change

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

None

7. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** *None*
- **QUAD CHARTS:** *N/A*

8. **APPENDICES:** *Attached and includes*

I. Combined PDF of (3) key full length published papers published this year.

Appendix

Journal of Trauma and Acute Care Surgery

Acute Resuscitation with Polyethylene Glycol-20k: A Thromboelastographic Analysis

--Manuscript Draft--

Manuscript Number:	
Full Title:	Acute Resuscitation with Polyethylene Glycol-20k: A Thromboelastographic Analysis
Article Type:	AAST 2018 Podium
Keywords:	Polymers; hemorrhage; shock; trauma; coagulation
Corresponding Author:	Niluka Wickramaratne, MD Virginia Commonwealth University Medical Center Richmond, VA UNITED STATES
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Order of Authors Secondary Information:	
Manuscript Region of Origin:	UNITED STATES
Opposed Reviewers:	

VIRGINIA COMMONWEALTH UNIVERSITY

VCU Medical Center

Ernest E. Moore, M.D.
Editor-in-Chief
Journal of Trauma and Acute Care Surgery
655 Broadway, Suite 365
Denver, CO 80204

September 4, 2018

Dear Dr. Moore:

Please find uploaded the manuscript entitled "*Acute Resuscitation with Polyethylene Glycol-20k: A Thromboelastographic Analysis*" that we are submitting to the Journal of Trauma and Acute Care Surgery for consideration for publication. This manuscript is an Original Article and the study type is Basic Science. This work has been accepted for a podium presentation at the upcoming American Association for the Surgery of Trauma 2018 Annual Meeting in the Basic Science section (Session XVB: Papers 30-38). This manuscript contains novel findings that have not been published elsewhere and will not be published elsewhere until a decision has been reached as to the applicability for the J. Trauma. All authors have significantly contributed to this study and to the preparation of the manuscript. All authors have approved the manuscript and agree with its submission to the Journal of Trauma and Acute Care Surgery. There are no conflicts of interest to disclose.

Thank you for this consideration

Sincerely,

Martin J. Mangino, Ph.D.
Professor

Medical College of Virginias Campus • Department of Surgery • Division of Acute Care Surgery

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ABSTRACT:

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 were hemorrhaged until the lactate reached 7 mmol/L or 50-55% of their estimated blood volume (EBV) was removed. A laparotomy was performed to simulate tissue injury. Low volume resuscitation (LVR) was given with FITC-labelled 10% PEG-20k solution (100mg/ml) or Lactated Ringers, both delivered at volumes equal to 10% of the EBV (n=5). Thromboelastography was performed after surgery (baseline), after hemorrhage, and 15, 120, and 240 minutes post-resuscitation. Survival time was measured and plasma PEG-20k concentration was determined by indicator dilution. All studies were terminated at 240 minutes due to the acute nature of the experiment.

Results

Pigs given PEG-20k survived 2.6-fold longer than controls ($p<0.001$), which was an underestimation due to arbitrary termination at 240 minutes. In controls, TEG data was limited to 15 minutes post-resuscitation due to lack of survival. Hemorrhage and injury induced a hypercoagulable trend on TEG, which was reversed with PEG-20k, primarily by affecting clot strength. This resulted in a neutral coagulation profile overall. The plasma concentration of PEG-20k peaked at 3.58 mg/ml, with a half-life of 59.6 minutes. The peak was 3-fold lower than predicted by simple dilution (10 mg/ml).

Conclusions

These data demonstrate that acute resuscitation with PEG-20k not only improves tolerance to hypovolemia but also normalizes the initial hypercoagulable state of injury. Although PEG-20k may interfere

with coagulation and platelet function above 10 mg/ml, peak plasma levels immediately after resuscitation in models of severe hemorrhagic shock are much lower than predicted with no clinically relevant effects on TEG.

Study Type

Original Article, Basic Science

Keywords

Polyethylene glycol; hemorrhage; shock; resuscitation; coagulation

Acute Resuscitation with Polyethylene Glycol-20k: A Thromboelastographic Analysis

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Running Head: Polymer effects on coagulation after trauma.

BACKGROUND:

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] Over the past few decades, best practices for fluid resuscitation in trauma have shifted away from liberal crystalloid administration to a paradigm of 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 large volumes of crystalloid.[2–5] However, accessing blood products can present a logistical problem in austere settings, such as during prehospital transport and in combat situations, due to storage requirements and limited resources. Furthermore, although the low cost and shelf-stable properties of traditional crystalloids are advantageous in these environments, they 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] Because hetastarch (Hextend ®) is the best available volume expander, it is still currently recommended in combat settings when blood is not available, despite its adverse effects.[6] Therefore, in the absence of blood products, there continues to be a need for an ideal low-volume resuscitative fluid, with minimal adverse outcomes.

Recently, polyethylene glycol polymers have proven to be efficacious in animal models of hemorrhagic shock and resuscitation. Our lab demonstrated that polyethylene glycol 20,000 Da (PEG-20k), was highly effective for low volume resuscitation 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 while a proportion

remains in the capillary space, creating two osmotic gradients that non-energetically move isotonic fluid from the intracellular and interstitial spaces into the capillaries. In addition to acting as a volume expander, PEG-20k also prevents ischemic cell swelling, thus preventing compression of the microcirculation, which allows for more effective capillary exchange and oxygen transfer.[12]

Given that both PEG-20k and starches share a large, polymeric structure, there is concern that PEG-20k may interfere with the coagulation system, as hetastarch does. 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). However, when the dose was reduced by 25%, these effects did not occur.[13] Because the highest in-vivo PEG-20k plasma concentration after intravenous infusions, and the associated biological effects on TEG, is dependent on the algebraic sum of many opposing forces, these ex-vivo 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, pre-clinical porcine model of severe hemorrhagic shock. A secondary objective was to determine peak plasma levels 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 (30-40kg, Archer Farms, Darlington, MD) were fasted the evening prior to the experiment, allowing access to water. Anesthesia was induced using an intramuscular injection of ketamine and xylazine and an intravenous injection of propofol. Endotracheal intubation was performed and anesthesia was maintained with inhaled isoflurane at 1-2% with room air (Narkomed 2 Ventilator, Drager, Lubeck, Germany). A circulating warm water pad was used to maintain a temperature of 37-38^{o} C (Gaymar T/Pump, Kent Scientific, Torrington, CT).

After appropriate anesthesia was achieved, femoral vessel cannulation was performed using a cut-down approach. The femoral arteries were cannulated bilaterally, and one line was connected to a hemodynamic monitoring system (Powerlab, ADInstruments, Boston, MA), while the other line was used for controlled hemorrhage. One femoral vein was cannulated for fluid administration. All vascular catheters were kept patent by flushing them with heparinized saline (5 units/ml). A midline abdominal incision was made to simulate soft tissue trauma. Both ureters were cannulated for real time urine collection. As soon as the femoral vein was cannulated, a 300 ml bolus of Lactated Ringer's fluid was given to all animals to offset the hemodynamic depression from the anesthetics and to normalize pre-hemorrhage 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

After a 10-15 minute baseline period, the pigs underwent controlled hemorrhage via the arterial line, with the mean arterial pressure (MAP) held at 30-40 mmHg, until either the lactate reached 7-8 mmol/L, 112 minutes of hemorrhage time had passed, or 50-55% of the animals estimated blood volume (EBV, calculated using 67 * body weight in kg) was removed, based on 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 30-40 mmHg. If the animal began to compensate and the MAP rose above 45, additional blood was removed at a rate of 2 ml/kg. 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 low volume resuscitation (LVR) was given with either a 10% PEG-20k solution (100mg/ml) in Lactated Ringer's containing a fluorescein isothiocyanate (FITC)-labelled PEG-20k marker (Nanocs, New York, NY) or Lactated Ringer's (n=5 in each group) over five minutes using the roller pump. In both groups, LVR was given at a volume equal to 10% of the animals EBV. The 10% EBV dose was chosen because it corresponds to giving an approximately 500 cc bolus to a 70 kg human with a blood volume of 5 L. Due to their acute nature, the experiments were stopped either when the animal died, or arbitrarily at 240 minutes after resuscitation was given. In addition to the coagulation measurements, other outcomes included the LVR Time, defined as the time from the start of LVR administration to the time the animal died or the lactated re-accumulated back to 7-8 mmol/L. If the lactate remained normal, the LVR Time was automatically 240 minutes. The mean arterial pressure and lactate value at the end of the experiment were also measured.

Animals that did not die from hemorrhage and shock were euthanized using intravenous potassium chloride administration. The shock and resuscitation protocol is shown in **Figure 1**.

TEG Assay

Whole blood thromboelastography was used to determine the effect of shock and low volume resuscitation 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, 120 and 240 minutes after resuscitation was given (TEG 5000 Haemoscope, Haemonetics, Braintree, MA). All blood samples were collected in sodium citrate tubes. Clot formation was initiated using re-calcification and kaolin activation. The TEG assays were completed using cups containing heparinase in all experiments except for the baseline measurements in the control group. The specific TEG parameters that were compared in this study included the reaction time (R time), which represents the time to clot initiation and is dependent on enzymatic coagulation factors. The α Angle and kinetics (K) time were measured, both of which represent clot kinetics and the rapidity of fibrin cross-linking. These factors are dependent on the concentration of fibrinogen present, such that a higher Angle and a shorter K time indicate faster clot propagation. The Maximum Amplitude (MA) was also measured. It represents the strength of clot formation and it is primarily due to platelet function. Finally, the Coagulation Index (CI) was also analyzed. The CI is a mathematical compilation of the TEG parameters, R time, K time, Angle and MA, representing the overall state of the coagulation system.

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 blood samples were spun to isolate plasma, and the concentration of the PEG-20k marker in both plasma and urine was quantified by direct measurement of the FITC fluor using a fluorescence plate reader (FL-800, BioTek, Winooski, VT) with an excitation wavelength of 485 nM and an emission wavelength of 520 nM. Plasma concentration data was 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.

Statistical Analysis

All statistical analysis was performing using GraphPad InStat for Windows (GraphPad Software, La Jolla California USA, 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 non-parametric Kruskal-Wallis ANOVA with the Dunn's multiple comparisons test for data within groups, across time. For comparisons between groups, the Mann Whitney U nonparametric test was used. Finally, the Wilcoxon rank-sum test was used to compare TEG data to normal ranges.

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, greater than 50% of the animal's total blood volume was removed, and hemorrhagic shock lasted 45 to 112 minutes.

PEG-20k increased the LVR time 2.6 fold over the LR control ($p < 0.001$). In the LR control group, the animals died 90 minutes after resuscitation (range 49 – 152 min), compared to 240 min in the PEG-20k group. The plasma lactate concentration 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 vs 2.2 mmol/L, $p = 0.6733$). All experiments in the PEG-20k group were all arbitrarily terminated at 240 minutes due to the acute nature of the experiments.

Coagulation

The coagulation and platelet function data is presented in **Figures 2 and 3.** At the baseline time point (BL), the R value data in the LR groups is not shown due to inaccuracy resulting from some heparin contamination in these blood samples. Analysis of the other parameters, K time, Angle, Maximum Amplitude (**Figure 2B-D**), and the Coagulation Index (**Figure 3**), demonstrates that there were no significant differences between the PEG-20k group and LR controls at the baseline time point (BL) and after hemorrhagic shock (HS).

Hemorrhage and surgical trauma caused a trend towards hypercoagulability, however, these changes were not statistically significant for each individual TEG parameter when comparing the post-hemorrhage 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 baseline (BL) in both groups. Again, these changes did not achieve statistical significance.

After low volume resuscitation was given, there were immediate effects on thromboelastography when comparing the post-resuscitation data across time (LVR15 – LVR 240) to the hemorrhage time point (HS) within the PEG-20k group (**Figure 2**). Specifically, administration of PEG-20k caused a decrease in the Maximum Amplitude (MA) from a post-hemorrhage (HS) mean of 70.64 ± 4.689 mm to a mean of 58.1 ± 4.794 mm at the LVR + 15 minute time point ($p < 0.05$). These values are still within the normal range. While PEG-20k did not cause statistically significant changes to the other TEG parameters at the 15 minute time point, the Coagulation Index (**Figure 3**) did decrease from 3.38 ± 1.163 post-hemorrhage (HS) to 0.1 ± 0.383 at LVR + 15 minutes ($p < 0.05$). Furthermore, 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 ($p < 0.05$). However, by LVR + 120 minutes, the MA began to recover from the initial effect of PEG and was no longer significantly different than the post-hemorrhage value (**Figure 2D**).

In the LR control group, data is 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 toward a hypercoagulable profile at LVR + 15 minutes, none were statistically different than their corresponding post-hemorrhage, pre-LVR value (HS). 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 smaller Angle (64.62 vs. 76.5, $p = 0.0082$), and a smaller 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**.

Plasma and Urine PEG Concentrations

The plasma and urine PEG concentration data are displayed in **Figure 5**. The peak plasma concentration of PEG-20k was 3.58 mg/ml 15 minutes after administration. The data was 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 (**Figure 5A**). Similarly, PEG-20k appeared in the urine within 15 minutes of administration and followed a similar decay pattern as the plasma concentration (**Figure 5B**).

DISCUSSION:

Our lab's previous studies have demonstrated the superior efficacy of a novel, polymer-based, low-volume resuscitative fluid that can extend tolerance to a hypovolemic state in animal models of hemorrhagic shock. Specifically, polyethylene glycol 20 kDa has been shown to non-energetically move fluid into the capillaries and counteract ischemic cell swelling. Not only does this provide lasting volume expansion that can be likened to an auto-bolus of isotonic fluid that was otherwise maldistributed in the extra-vascular space due to injury, but it also decompresses the microcirculation. The resulting improvement in convective oxygen transfer allows for better repayment of oxygen debt, lactate clearance, and survival compared to colloids and crystalloids.[10–12] Clinically, PEG-20k based resuscitative fluids would be ideal for prehospital use when blood is impractical because the improved metabolic and cardiovascular tolerance to shock could allow for much longer transport times, without the adverse effects of other fluids. Continued development of these solutions for clinical use require that side effects and contraindications be identified.

The basis for the current study was a previous experiment 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 thromboelastography. The ex-vivo 10% volume dilution was chosen because it mirrored the volume administered in our animal models of hemorrhagic shock, representing the higher range of PEG-20k dosing for low-volume resuscitation. The ex-vivo data showed that the 10% dose of PEG-20k affected both clot propagation (increased K time, decreased Angle) and clot strength (decreased MA). In fact, the reduction in MA, which primarily represents a defect in platelet function, would likely be significant enough to trigger a transfusion based on the ACS TQIP Massive Transfusion in Trauma Guidelines (MA < 55mm). 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. This is important because we know that the hypocoagulable effects of PEG-20k on ex-vivo blood clotting are dose-dependent and may be lost just below 10 mg/ml (the theoretical peak blood level in patients after a 10% blood volume IV infusion of a 100 mg/mL solution). Therefore, the objective of the current study was to determine the effects of low volume resuscitation with PEG-20k on coagulation and platelet function using TEG in a clinically relevant model of hemorrhagic shock; and to correlate this to the pharmacokinetics and peak blood levels of PEG-20k in shocked animals, which is largely unknown.

In general, the TEG data suggest that our model detected a hypercoagulable state after hemorrhage and trauma. This persisted in the control group until the animals died of shock, but it

was reversed by resuscitation with PEG-20k. Despite this, the coagulation and platelet function data were unremarkable and within normal ranges after PEG-20k administration. The significant changes in MA, Angle, and K time observed previously in the ex-vivo blood tests[13] were not observed in this pre-clinical, in-vivo model. There were early changes in MA 15 minutes after PEG-20k administration that were similar to what was observed ex-vivo, but the values remained within normal ranges, and this effect normalized at later time points. 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. The most likely explanation is a difference in blood levels when the solution was administered IV compared to the theoretical 10% dilution assumed in the ex-vivo whole blood tests. In fact, the blood levels were about 3 fold lower in-vivo compared to what was used ex-vivo. This indicates that not only are the PEG-20k solutions highly effective for resuscitation but they are also safe for normal clot formation in trauma patients. This data supports our hypothesis that the effect of PEG-20k on coagulation is dependent on plasma concentration.

After severe injury, hemostasis becomes deranged due to a complex interplay between multiple systems, involving neuroendocrine, inflammatory, and coagulation pathways. The resulting effect on the patient as a whole may be a hypercoagulable or hypocoagulable state, which is modulated by the severity of injury, as well as other patient-specific factors. Therefore, using PEG-20k for acute resuscitation may provide added value by reversing the hypercoagulable state that is known to occur in a subset of trauma patients early after injury and treatment.[15–20] This could aid in preserving tissue perfusion in vital organs and decrease the incidence of venous thromboembolic disease in the surgical-trauma ICU.

The reason there was no major effect of PEG-20k on TEG in this study, compared to the earlier study in whole blood, seems to be a dose effect. The optimal dose of PEG-20k in shock

models was a 10%, or 100 mg/mL, PEG-20k solution given IV at a volume equal to 10% of the estimated blood volume, which is a theoretical 10 fold dilution of the infusate, or 10 mg/ml. Therefore, this concentration was used in the ex-vivo whole blood TEG study where significant TEG effects were observed. While this concentration was assumed to closely approximate blood levels in-vivo, the actual peak blood levels of PEG-20k observed after IV infusion in pigs show concentrations of only about 3.5 mg/ml. The 3 fold lower blood levels in-vivo likely explains why TEG effects were not observed.

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 1/3 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. This seems to protect from coagulopathies.

Even though the values remained within normal ranges, the effect of PEG-20k on the MA was statistically different than the pre-LVR values and the LR control group 15 minutes after resuscitation. However, this effect 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 PEG-20k 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.[21–23] This would also explain the effects of PEG-20k on clot propagation, which may be due to decreased access to the catalytic surface on the platelet membrane. Further mechanistic studies are required to delineate the exact mechanism.

In summary, this study demonstrated that when given at doses that are highly effective for low volume resuscitation of severe hemorrhagic shock, PEG-20k had no effects on TEG outcomes except to limit a slight hypercoagulable state associated with trauma. Pharmacokinetic data demonstrated a lower than expected peak plasma concentration and rapid clearance, which is responsible for the lack of TEG effects observed in-vivo after shock resuscitation.

FIGURE LEGENDS:

Figure 1: The experimental design of the project using two groups of anesthetized juvenile pigs (n=5 each). After anesthesia, the 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, labs, 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 7-8 mmol/L, blood loss of 50-55% of total blood volume, or a shock time of 112 min. Following the trigger, low volume resuscitation (LVR) is administered at a volume of 10% of the estimated blood volume, given IV over 5 min. Either

lactated ringers (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.

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 = open circles and PEG treated = black squares). The blood samples from each pig in both groups were analyzed at Baseline (BL), after Hemorrhagic Shock (HS), and after low volume resuscitation (LVR) for 15, 120, 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 normal range; # - R data in the LR group at baseline was excluded due to inaccuracy. The hatched area represents the normal values for the various TEG parameters for human blood.

Figure 3: TEG values for the CI (coagulation Index) are shown for the two groups of pigs (LR = open circles and PEG treated = black squares). 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 Baseline (BL), after Hemorrhagic Shock (HS), and after low volume resuscitation (LVR) for 15, 120, 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 normal range. The hatched area represents the normal values for CI for human blood where > +3.0 is hypercoagulable and < -3.0 is hypocoagulable.

Figure 4: Thromboelastograph (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), and 15 minutes after low volume resuscitation (LVR + 15 min). The hypercoagulable state after hemorrhage can be seen as a large MA, fast K time, and steep Angle after hemorrhage, relative to the baseline tracing. These hypercoagulable attributes remain in the pigs resuscitated with low volume LR solution but a clear reversal of this state is seen with low volume resuscitation using PEG-20k solution.

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 (A). The rapid clearance of PEG-20k can be seen by the rapid appearance and disappearance of FITC-PEG in the urine (B), which is the major route of elimination of the polymer . All data points represent the average of 5 independent pig studies.

AUTHOR CONTRIBUTIONS:

Each author has contributed significantly to and is willing to take public responsibility for aspects of this study: NW, MA, and MM contributed to the literature review study design. NW, KK, HR, CB, RL, MA and MM performed the data acquisition. NW and MM performed the analysis and interpretation of data and wrote the manuscript.

DISCLOSURE:

The authors have no conflicts of interest to declare.

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TABLES:

Table 1:

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

	Control: LR	**Treatment: 10% PEG-20k**
Weight (kg)	34.32 (5.12)	29.4 (2.23)
Estimated blood volume (mL)	2299.4 (342.3)	1969.5 (150)
Baseline MAP (mmHG)	79.6 (10.5)	70.8 (3.7)
Baseline lactate (mmol/L)	1.9 (0.46)	1.92 (0.64)
Hemorrhage 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 (mmHg)	N/A ǂ	56.6 (5.9)

Data expressed as means (standard deviation) | * $p < 0.001$ | ǂ No terminal MAP data - animals expired

Figure 1

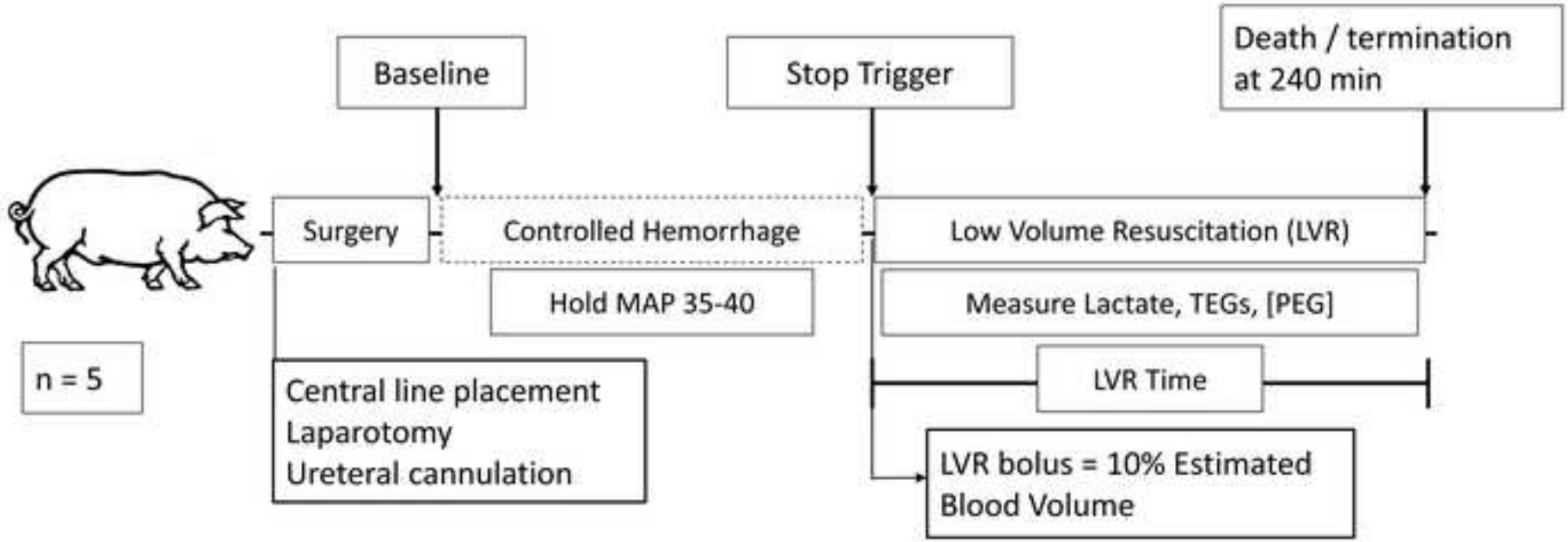


Figure 2

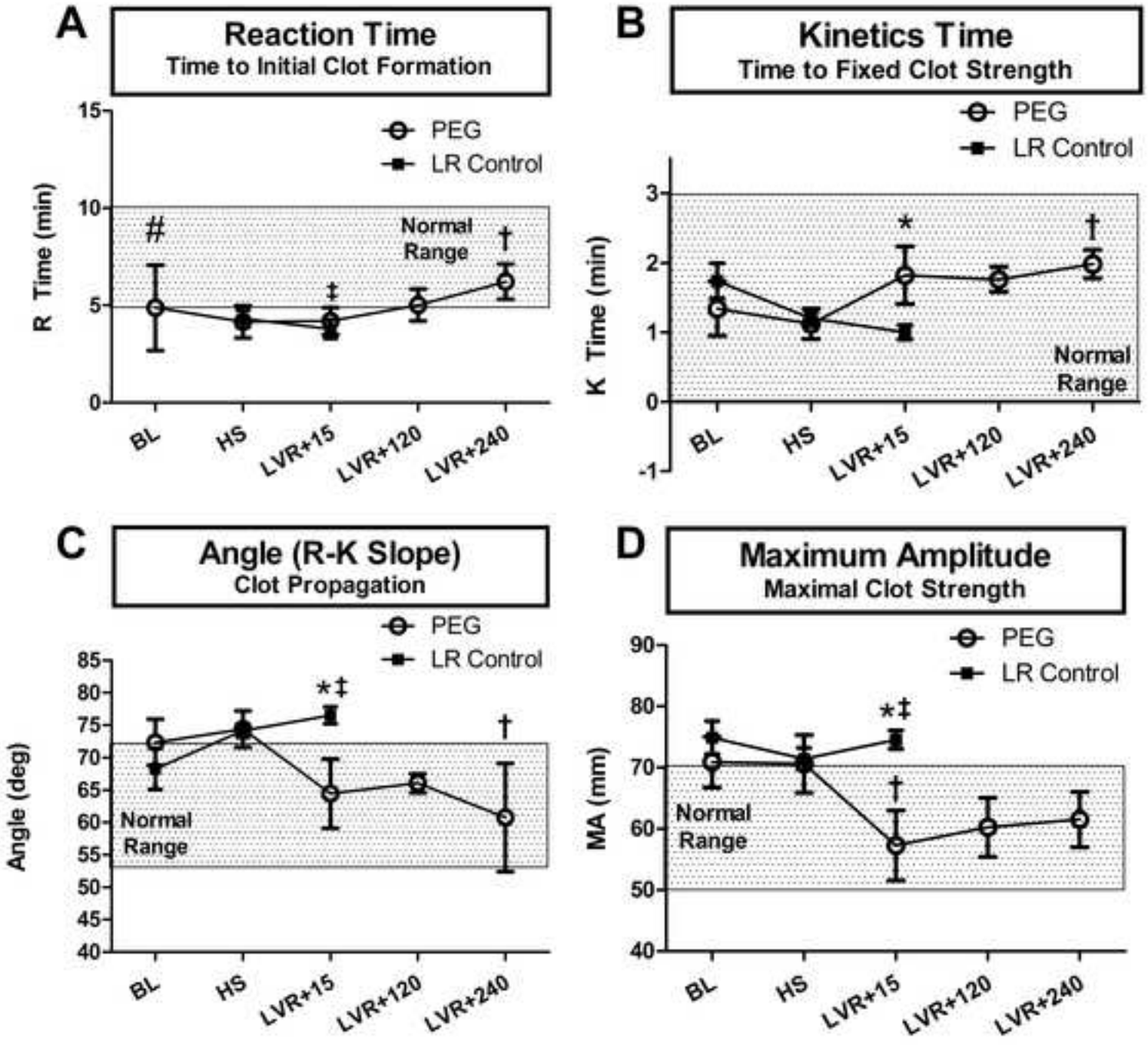


Figure 3

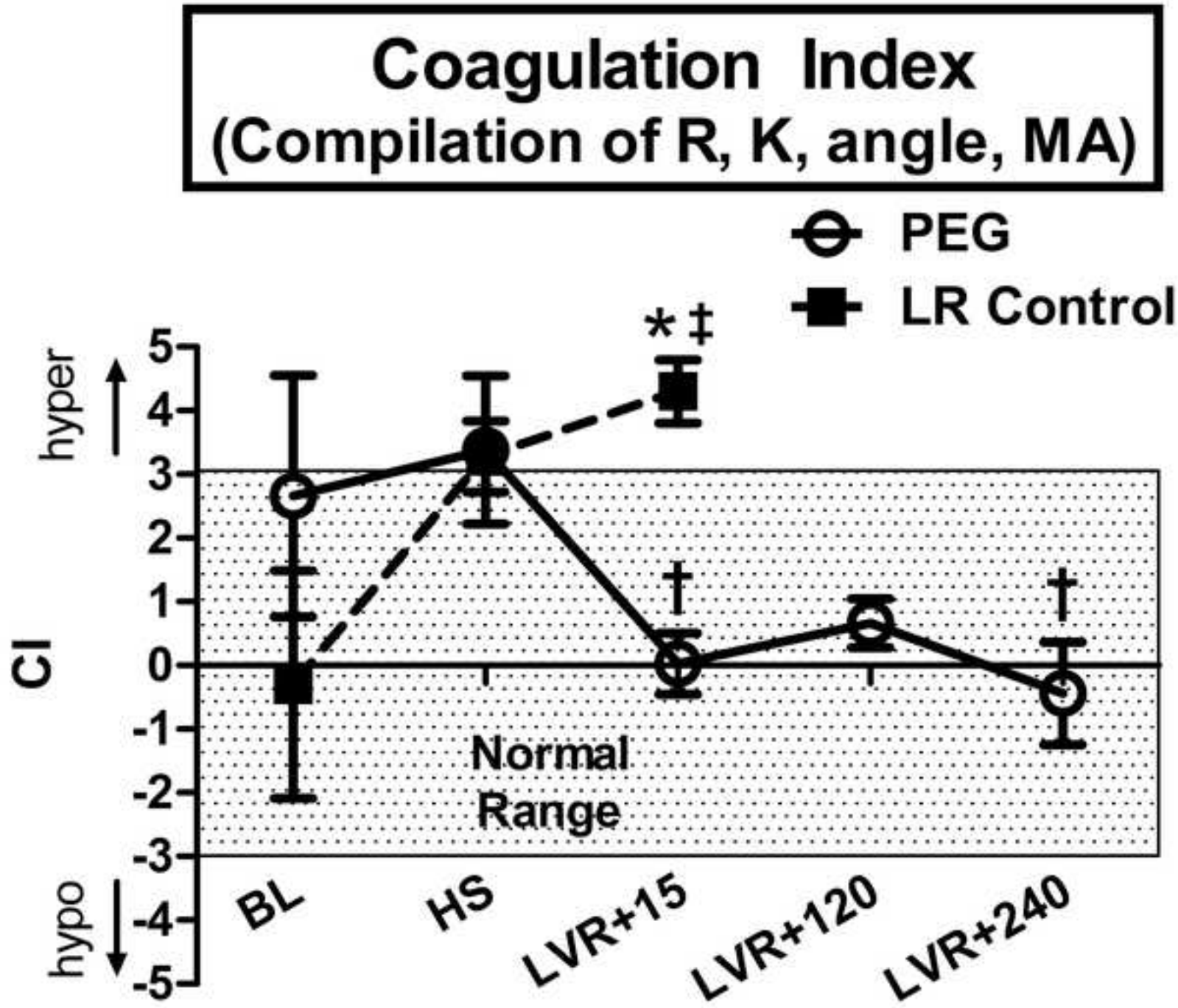


Figure 4

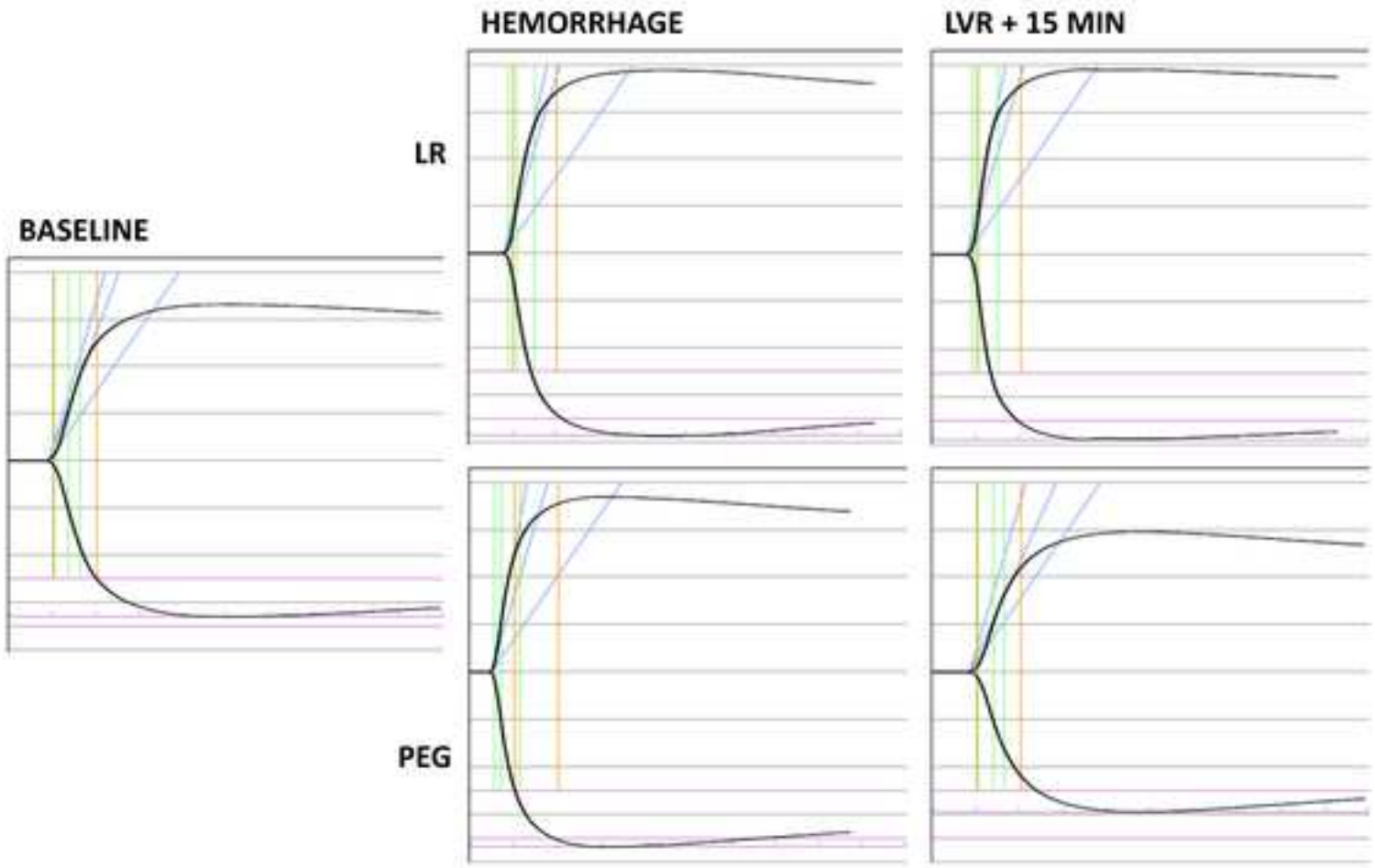
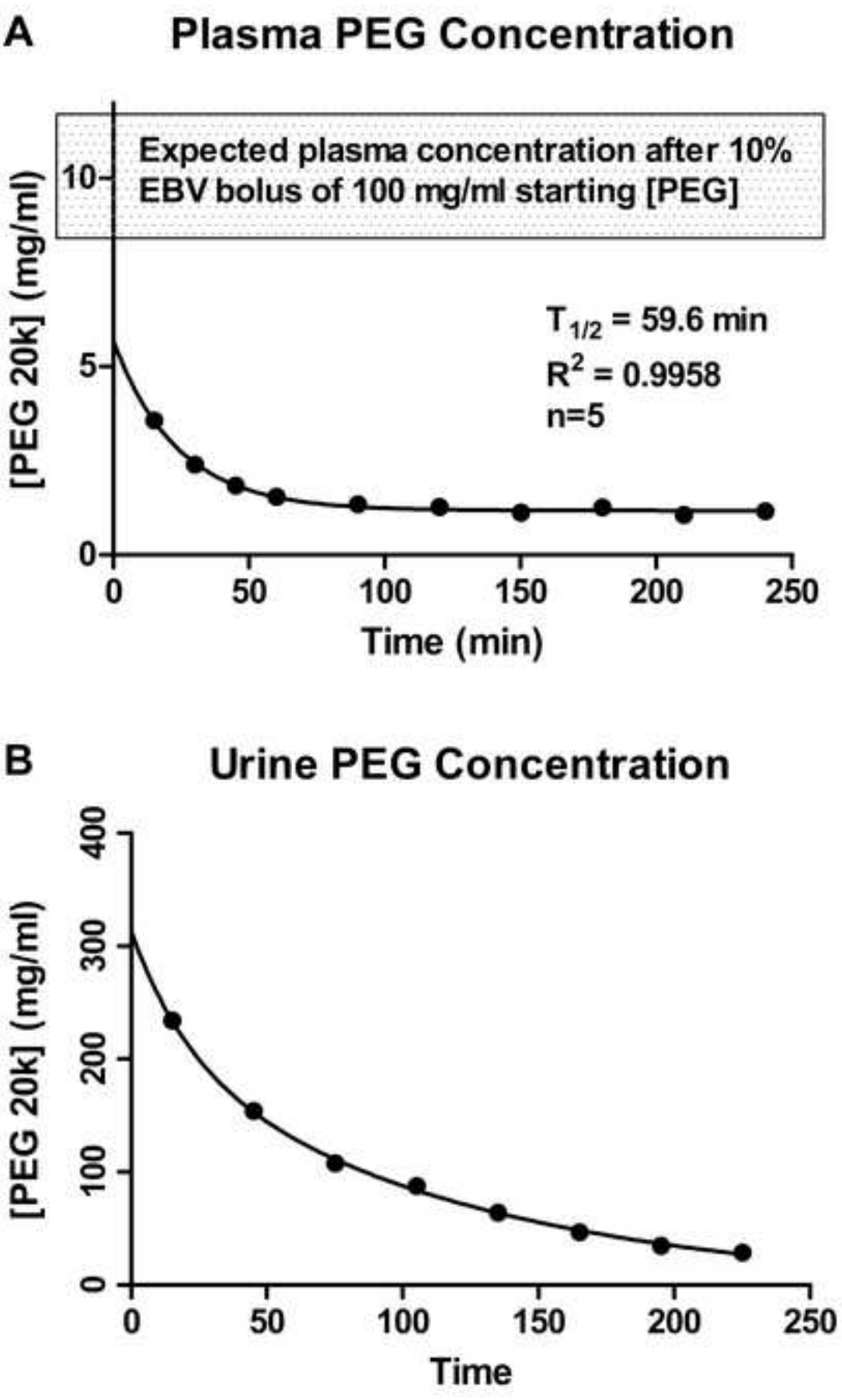


Figure 5



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Thromboelastographic analysis of novel polyethylene glycol based low volume resuscitation solutions

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Full Title:	Thromboelastographic analysis of novel polyethylene glycol based low volume resuscitation solutions
Short Title:	Clot formation after polymer based crystalloids
Corresponding Author:	Martin J Mangino Virginia Commonwealth University School of Medicine Richmond, VA UNITED STATES
Keywords:	pre-hospital damage-control hemorrhagic shock coagulopathy trauma TEG coagulation fluid
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). 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.
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All animal work must have been conducted according to relevant national and international guidelines. If your study involved non-human primates, you must provide details regarding animal welfare and steps taken to ameliorate suffering; this is in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research." The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement. If anesthesia, euthanasia or any kind of animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied. Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained. **Field Permit** Please indicate the name of the institution or the relevant body that granted permission.	
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Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?	
Please describe where your data may be found, writing in full sentences. **Your answers should be entered into the box below and will be published in the form you provide them, if your manuscript is accepted.** If you are copying our sample text below, please ensure you replace any instances of XXX with the appropriate details. If your data are all contained within the paper and/or Supporting Information files, please state this in your answer below. For example, “All relevant data are within the paper and its Supporting Information files.” If your data are held or will be held in a public repository, include URLs, accession numbers or DOIs. For example, “All XXX files are available from the XXX database (accession number(s) XXX, XXX)." If this information will only be available after acceptance, please indicate this by ticking the box below. If neither of these applies but you are able to provide details of access elsewhere, with or without limitations, please do so in the box below. For example: “Data are available from the XXX Institutional Data Access / Ethics Committee for researchers who meet the criteria for access to confidential data.” “Data are from the XXX study whose authors may be contacted at XXX.” * typeset	Data are available from the corresponding author by request.
Additional data availability information:	

VIRGINIA COMMONWEALTH UNIVERSITY

VCU Medical Center

Dr. Joerg Heber
Editor-in-Chief
PLoS1

June 21, 2018

Dear Dr. Heber:

Please find uploaded the revised manuscript entitled "Thromboelastographic Analysis of Novel Polyethylene Glycol Based Low Volume Resuscitation Solutions" that we are submitting for consideration for publication in PLoS1 as a regular research article. This manuscript is the first of two that we are submitting at the same time and we request that they be considered and published as companion papers because of their significance and sequentially linked content and interest. The second paper is entitled "Mechanisms of Novel Polyethylene Glycol Based Low Volume Resuscitation Solutions on Coagulation and Platelet Function", which will be uploaded subsequent to this one. While sequentially related, each paper is independent and stands on its own weight. This study contains original data that have not been published before and will not be considered for publication elsewhere until a final decision has been made regarding its acceptability to PLoS1. This study describes the effects of a novel and highly effective new low volume resuscitation solution used to resuscitate patients in severe hypovolemic shock on overall coagulation and platelet function using Thromboelastography. This is of interest since previous polymer based IV solutions caused severe coagulopathies. The companion paper produced subsequently describes the potential mechanisms of the observed thrombasthenia in the first report. This manuscript was approved by all authors and all authors contributed significantly to the study without conflicts of interest. We suggest Dr. Scott Brakenridge at UF, Gainsville, FL, USA as an appropriate and highly qualified academic editor for this paper.

Thank you for this consideration.

Respectfully,

Martin Mangino
Professor and Associate Chair

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Thromboelastographic analysis of novel polyethylene glycol based low volume resuscitation solutions

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Short Title: Clot formation after polymer based crystalloids

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).

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 crystalloid volume stays in the intravascular space and 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 particularly pertinent differences may also include vascular interactions with the glycocalyx and capillary structures in varying shock and ischemic states (11, 12).

Intravenous administration of Hextend and 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 whole blood coagulation and platelet function in both healthy volunteers and a selection of trauma patients, and to identify any existing mechanisms of such coagulopathies.

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. 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. 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:

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 Figure 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.

Amplification of Clotting: The TEG k parameter is shown in Figure 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: Figure 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 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 Figure 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-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 Figure 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 figure 6 for the blood dilutions in the healthy volunteers, the trauma patients, and the volunteers in the PEG-20k dose-response study. The coagulation index (CI) is a mathematical compilation of other TEG parameters and is described by $CI = -0.3258R - 0.1886K + 0.1224MA + 0.0759\alpha - 7.7922$. The normal range for CI is between 3.0 and -3.0. As shown in Figure 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). Figure 7 shows that reducing the 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:

i. 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 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 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.

ii. 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 (data not shown). 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.

iii. 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). Actually, k and angle seem to be most severely affected over any other parameter. 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. Based on the TEG data, the polymer may create a state of functional thrombocytopenia, as opposed to a physical thrombocytopenia, because

MA is lowered after matched dilutions with PEG-20k and starch, as opposed to saline, which all began with the same number of platelets and blood in their respective test tubes.

Without further testing, it is not possible to know for sure how PEG-20k polymers affect whole blood coagulation, but our preliminary hypothesis at this time is that the polymer causes a state of reversible platelet thrombocytopenia through physical platelet passivation effects, maybe by cross linking or adsorbing platelets. This would functionally remove platelets from the system and prevent their direct aggregation to form a platelet-fibrin clot and reduce the membrane and phospholipid effects of the platelet on the acceleration of coagulation reactions and the rate of fibrin formation. Further mechanistic experiments to sort out these possibilities have been conducted and the results, analysis, and conclusions are presented in the follow on companion paper.

iv. 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 ≥ 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 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.

v. 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 a physical platelet passivation response is occurring. The clinical effects will need to be verified in an in-vivo model.

ACKNOWLEDGEMENT

FIGURE LEGENDS

Figure 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.

Figure 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

429 the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest
430 values, respectively. The shaded box represents the known normal ranges for the TEG values. WB=
431 whole blood (undiluted). *P<0.05.
432
433 **Figure 3**: Clot propagation indexed by the **Angle** variable on TEG in blood from healthy volunteers,
434 trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25
435 healthy volunteers, 9 trauma patients, and 9 volunteers for dose-response studies. Whole citrate
436 preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS),
437 and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full
438 thromboelastography (TEG) within 2-3 hours of blood draw in a matched design with saline always
439 serving as control to the resuscitative fluid. All values are expressed in a box and whiskers standard
440 format where the bar in the box is the sample median value, the lower border of the box is the value
441 demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating
442 the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest
443 values, respectively. The shaded box represents the known normal ranges for the TEG values. WB=
444 whole blood (undiluted). *P<0.05.
445
446 **Figure 4**: Clot strength and maximum size indexed by the **MA** on TEG in blood from healthy
447 volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes
448 represents 25 healthy volunteers, 9 trauma patients, and 9 volunteers for dose-response studies. Whole
449 citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend
450 (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full
451 thromboelastography (TEG) within 2-3 hours of blood draw in a matched design with saline always
452 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.

Figure 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)

Figure 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

Figure 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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Figure 1

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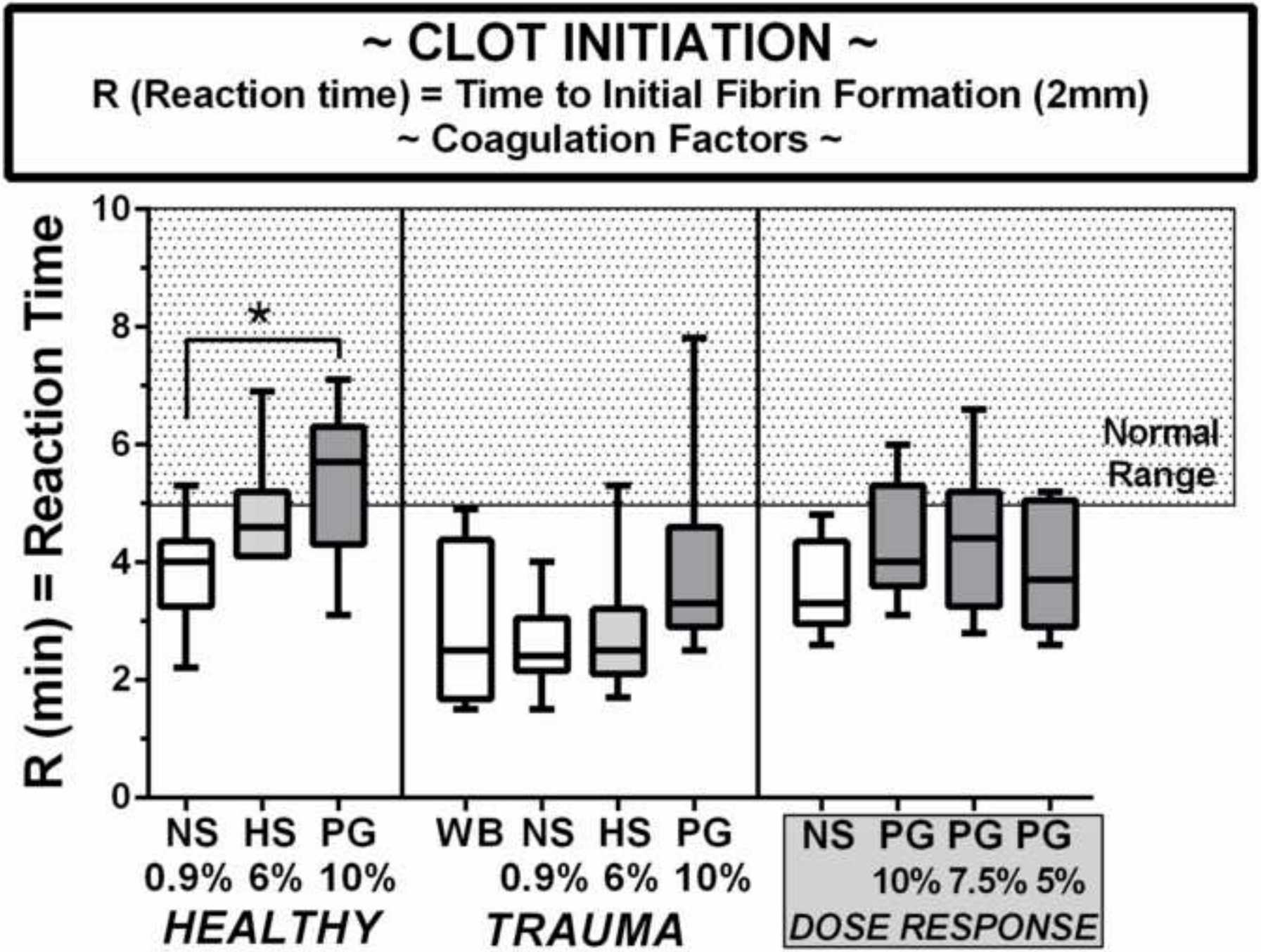

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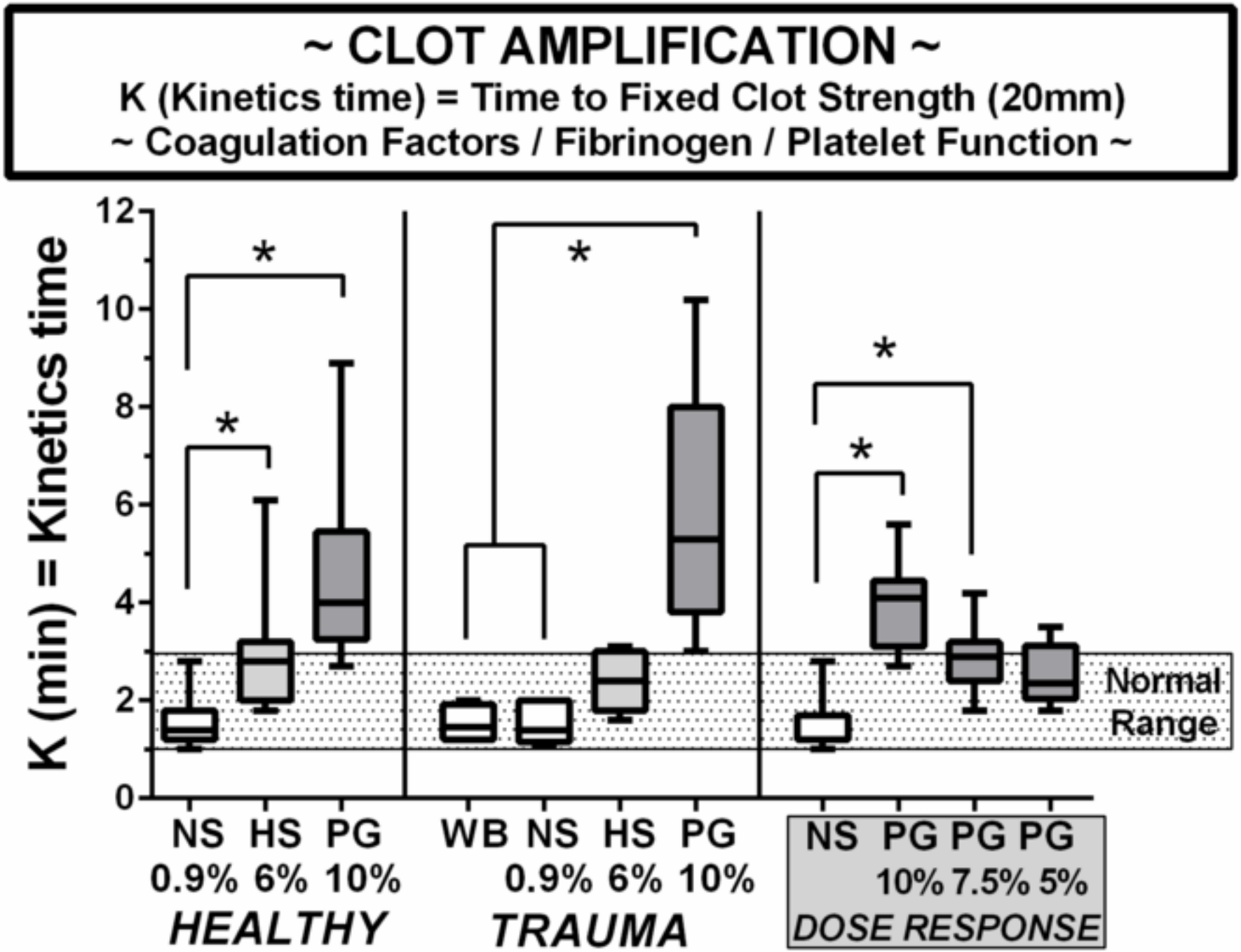

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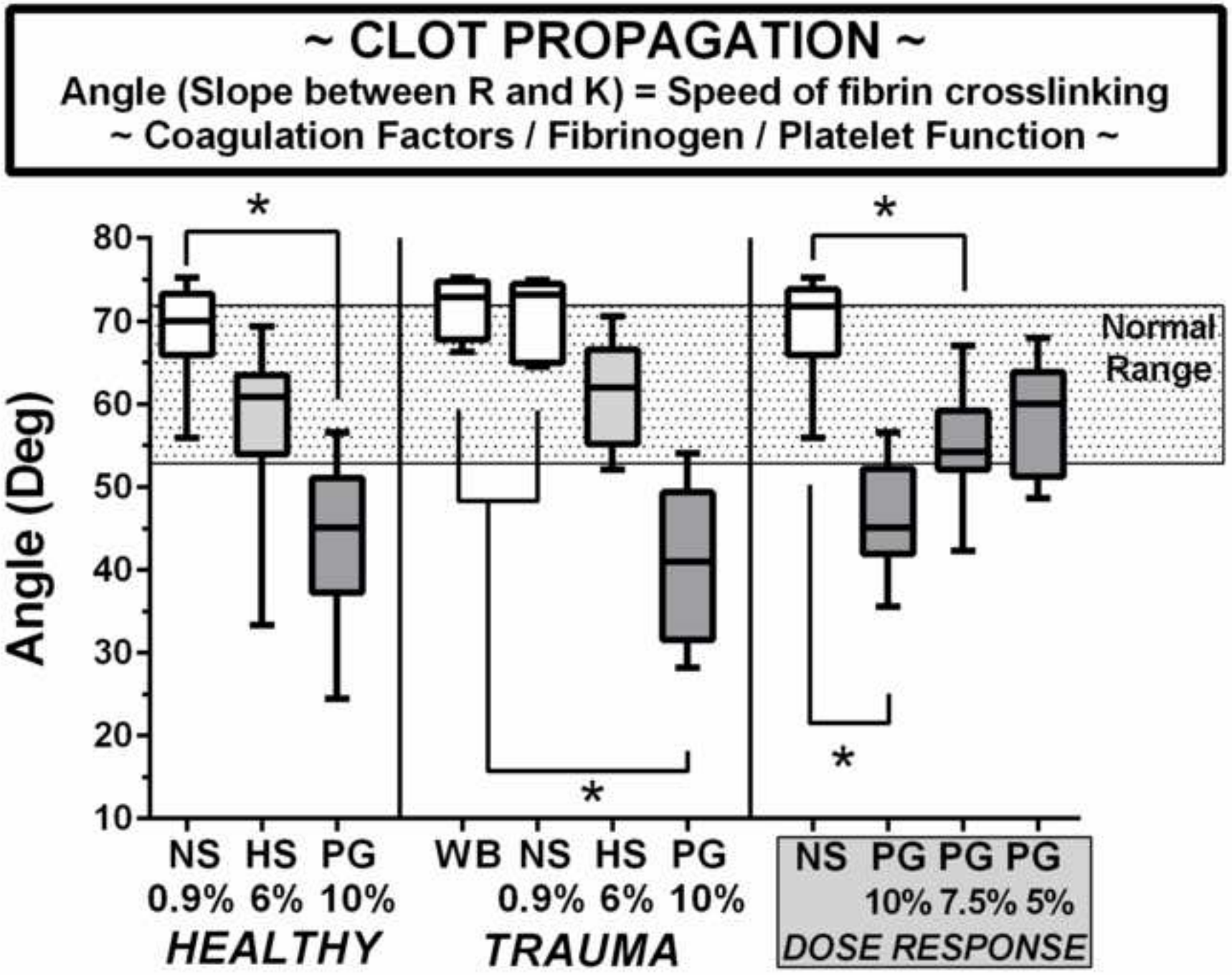

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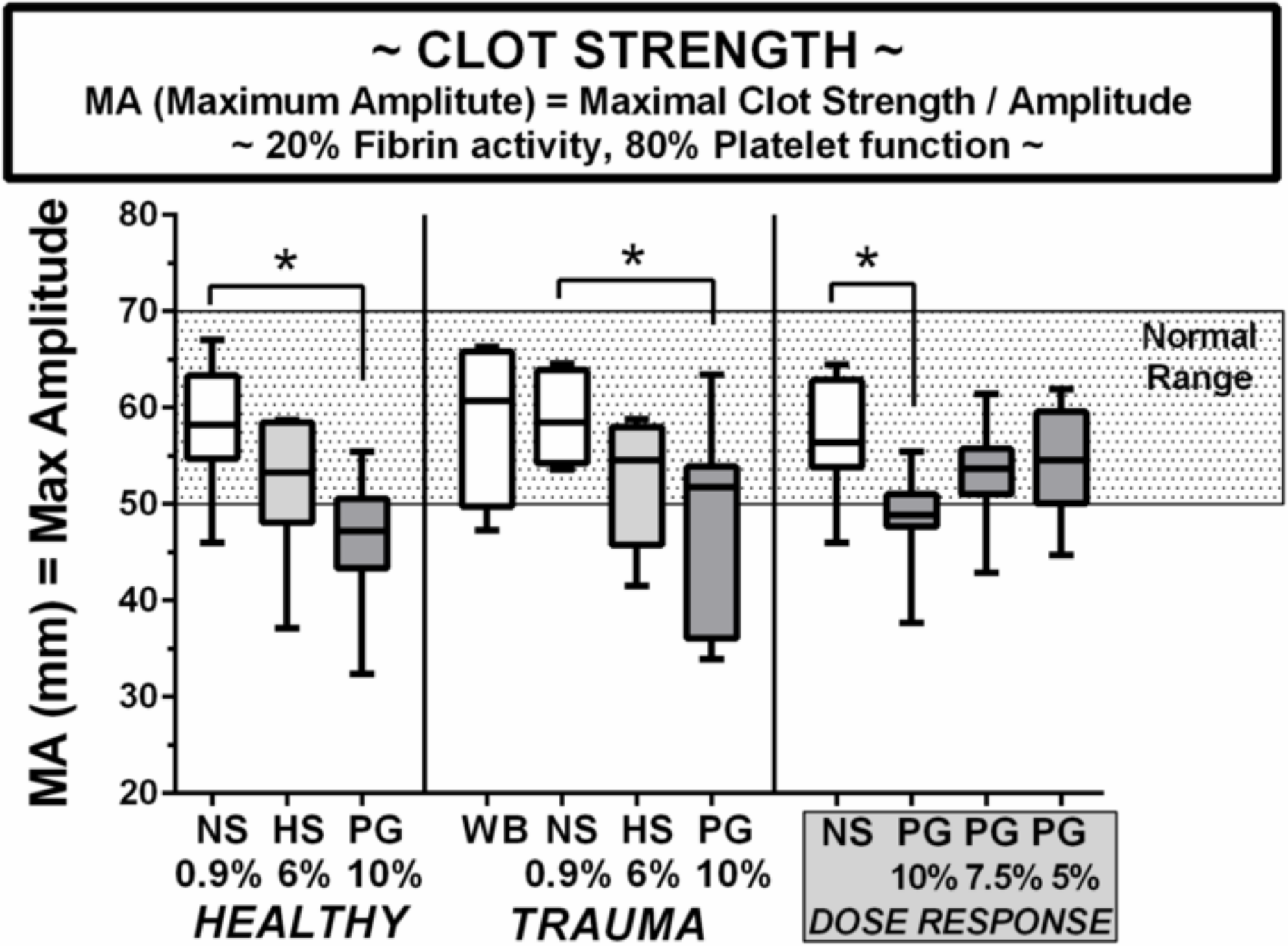

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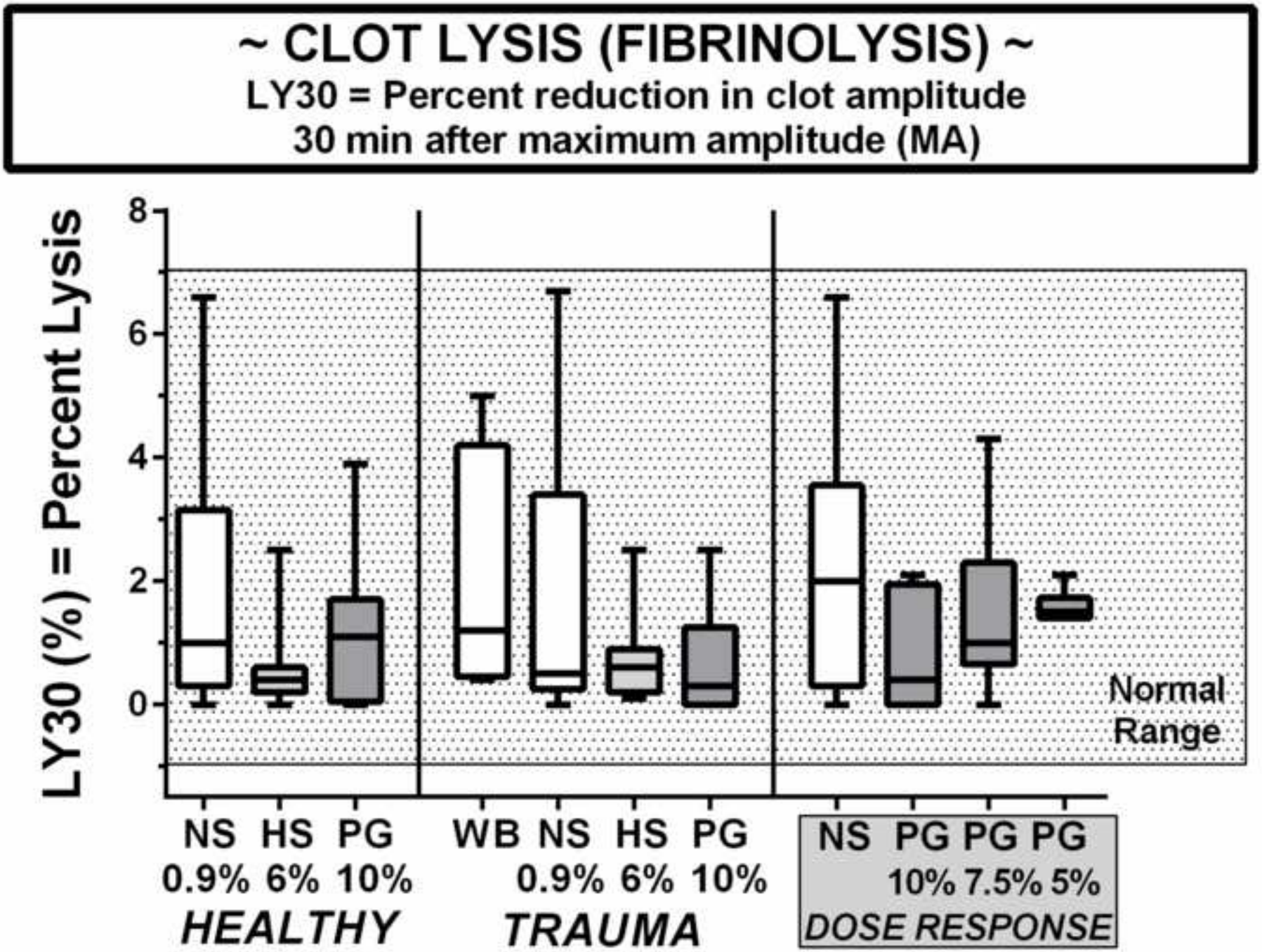

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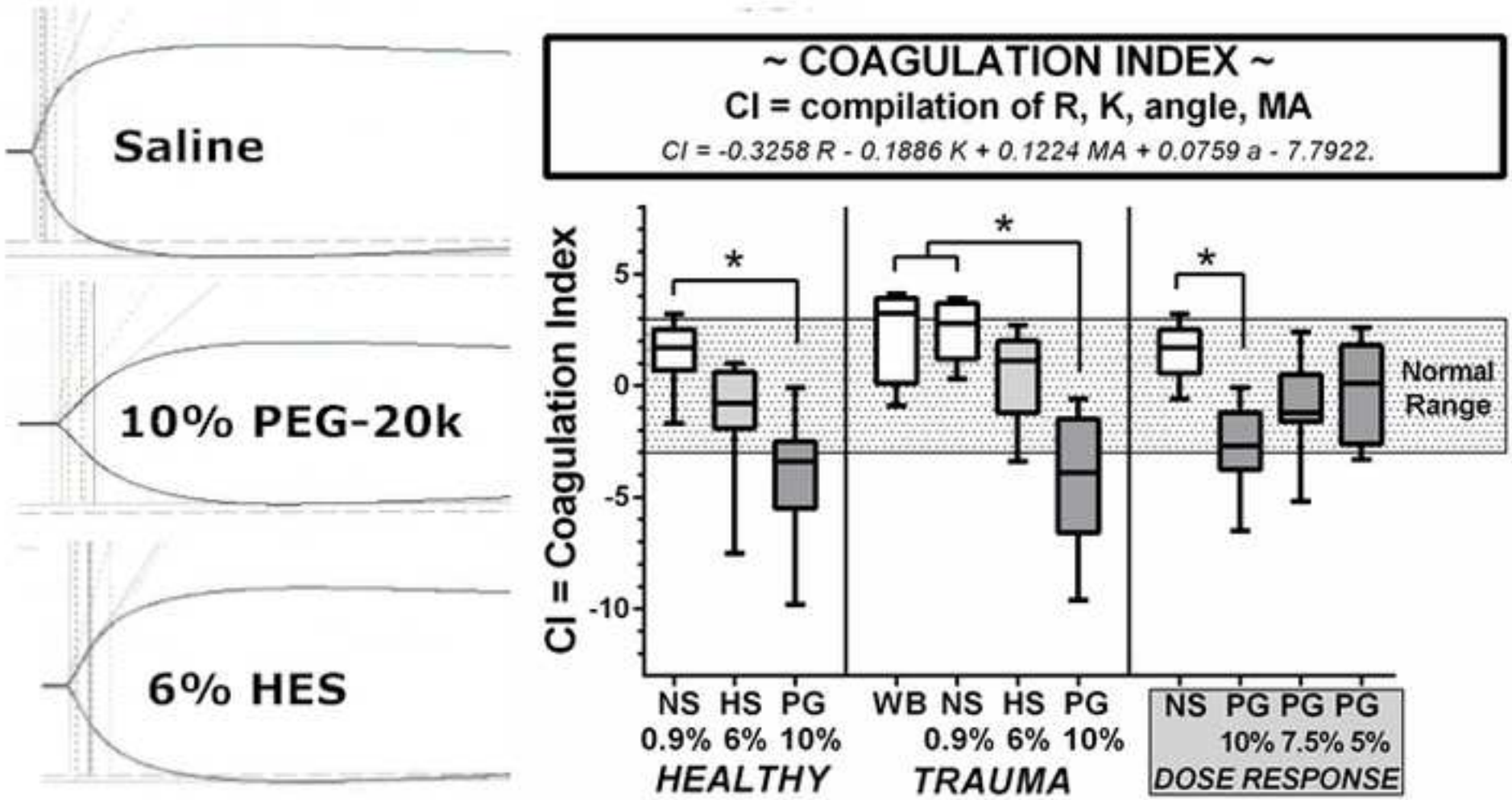

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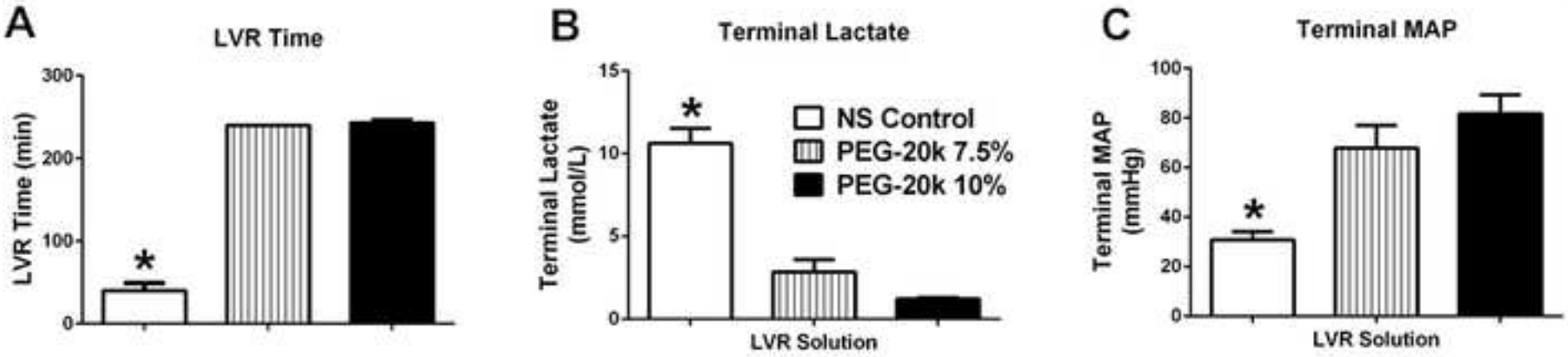

Mechanisms of novel polyethylene glycol based low volume resuscitation solutions on coagulation and platelet function*

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Running Title: Sites of polymer interference in clot formation.

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Abstract:	249
Introduction:	679
Discussion:	1,069

Abbreviations:
FITC, Fluorescein Isothiocyanate
LVR, Low Volume Resuscitation
PEG-20k, Polyethylene Glycol 20,000 Da
TEG, Thromboelastography
TICS, Trauma-Induced Capillary Leak Syndrome

Section Assignment Recommendation: Cardiovascular

ABSTRACT

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. 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 concentrations (vWf), thrombin generation, thromboelastography with platelet mapping, platelet flow cytometry, and erythrocyte sedimentation rate. Fibrinogen and vWF concentrations, PT, and aPTT were not affected by PEG-20k dilutions relative to saline dilution controls. Thrombin activity was mildly suppressed with PEG-20k dilution (TTP- 20%) relative to saline control. Platelet mapping demonstrated significantly greater % inhibition of both ADP and arachidonic acid-induced platelet aggregation with PEG-20k, compared to dilutional controls, but direct ADP-activated gpIIa/IIIb (PAC1) and P-selectin (CD62P) binding site expression was not altered by PEG-20k using flow cytometry. Erythrocyte Sedimentation Rates (ESR) were dramatically accelerated after dilution with 10% PEG-20k, compared to controls, and this was competitively blocked by smaller PEG polymers, suggesting nonspecific PEG-20k cell binding effects. PEG-20k creates a mild hypocoagulative state in whole blood at concentrations ≥10%, which may be due to platelet-PEG interactions at the IIb/IIIa interface, and not due to coagulation proteins defects. 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%) (Finkelstein et al., 2006). 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 (Kauvar et al., 2006). Hemorrhagic hypotension exposes the patient to immediate complications of life-threatening infections, coagulopathies, and multiple organ failure (Heckbert et al., 1998; Franklin et al., 2000).

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 (van Lambalgen et al., 1990; Parrish et al., 2015). 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 exacerbates 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; Riha et al., 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 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 (Parrish et al., 2015; Parrish et al., 2016; Plant et al., 2016; Plant et al., 2017). 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 (Plant et al., 2017). This causes rapid clearance of lactate, increased blood pressure, and tolerance to the low volume state (Plant et al., 2016). While these polymers work several-fold better than hydroxyethyl starch based polymers, implying different mechanisms of action, interference with blood clotting and coagulation may be shared by both types of polymers. For example, the IV starch-based crystalloid solutions Hextend® and Hespan® are complicated by both renal toxicity and coagulopathies (Mangino et al., 2017), which in trauma settings are a concern.

In a set of experiments recently published (Liebrecht et al., In Press), 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 both dose-dependent and related mainly to interferences of the polymer with platelet function.

MATERIALS AND METHODS

Volunteer Blood: Whole blood (15-ml) was drawn into citrated vacutainer collection tubes from 6 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 (Oswald et al., 1983; Blann, 1990; Hemker et al., 2003; Scarpelini et al., 2009).

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 (Hemker et al., 2003). 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 (Bochsen et al., 2007). Briefly, heparinized blood treated with reptilase and factor VIII was used to isolate only the fibrin component of clot formation; and 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.

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 (Schmitz et al., 1998). 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 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) obtained from healthy volunteers that had been diluted 10% with either PEG-20k (10% w:v) or a saline dilution control are shown **Figure 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 observed for the PEG-20k (Panel B) samples but the levels in this group were still within the normal range.

Figure 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 (Gu et al., 2014; Murphy et al., 2014; Hillarp et al., 2017). This is an artifact that should be avoided when testing PEG-20k diluted blood.

Figure 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.

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 **Figure 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 **Figure 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).

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 **(Figure 6**). 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).

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 (Liebrecht et al., In Press) 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 with platelets (80%) and fibrin (20%), it was posited that PEG-20k effects on coagulation may interfere with platelet function and/or fibrin polymerization. Deficiencies in fibrin polymerization were suspected given the decreases in the fibrinogen dependent TEG factors such as α-angle and k. These changes are affected by low fibrinogen activity, fibrinogen deficiency, or thrombocytopenia/thrombocytopathy. Therefore, to dissect out effects of PEG on coagulation proteins or platelet function, we conducted more specific testing on diluted volunteer whole blood.

This study essentially rules out PEG20k-related effects causing coagulation protein deficiencies. For example, the levels of fibrinogen were not different with PEG-20k diluted blood compared to the saline controls, and the fibrinogen remained within the normal ranges. The vWF concentrations were slightly lower in the PEG-20k spiked plasma samples, but also remained in the normal range. Therefore, the slower clot propagation and decreases in α-angle and k observed on TEG are not the result of lack of plasma fibrinogen or vWF. This likely rules out any potential interference or chemical interaction of PEG polymers with either fibrinogen or vWF. 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 PEGylated factor replacement products (Gu et al., 2014; Murphy et al., 2014; Hillarp et al., 2017)**.** 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 (Hemker et al., 2003). 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, Figure 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 (Liebrecht et al., In Press), 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. 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 (Israelachvili, 1997; McNamee et al., 2007; Wee et al., 2008; Sauter et al., 2013). Further studies

using fluorescent or electron microscopy imaging may be useful to resolve any potential platelet-PEG-20k pharmacodynamic interactions 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 observed in healthy volunteers and trauma patients. PEG-20k has little 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 platelet activation and clotting may 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.

AUTHORSHIP CONTRIBUTINS:

Participated in research design: Liebrecht, Newton, Martin, Wickramaratne, Brophy, Mangino

Conducted experiments: Liebrecht, Martin, Han, Mangino

Contributed new reagents or analytic tools:

Performed data analysis: Liebrecht, Newton, Martin, Wickramaratne, Brophy, Mangino

Wrote or contributed to the writing of the manuscript: Liebrecht, Newton, Martin, Wickramaratne, Jayaraman, Han, Aboutanos, Brophy, Mangino

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FOOTNOTES

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FIGURE LEGENDS

Figure 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.

Figure 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.

Figure 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.

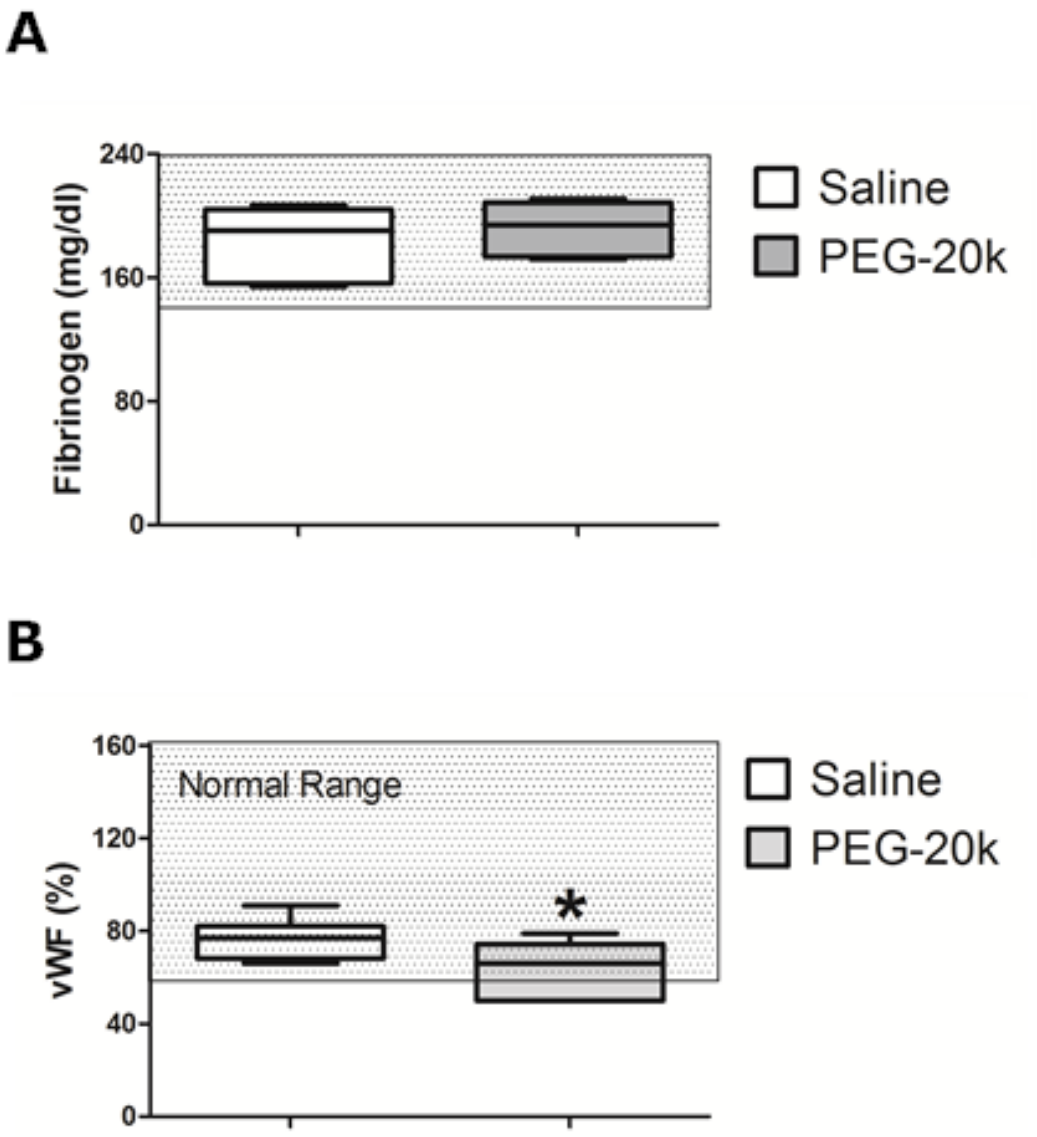
Figure 4: TEG platelet mapping studies conducted with whole blood obtained from volunteers diluted with either saline (1 to 9) or 10% PEG-20k solution (1 to 9). The figures shows the inhibition of activation of platelet clot formation in response to either ADP or arachidonic acid. The bar inside of the 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.

Figure 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.

Figure 6: 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

Figure 1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B
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vWF (%)
Normal Range
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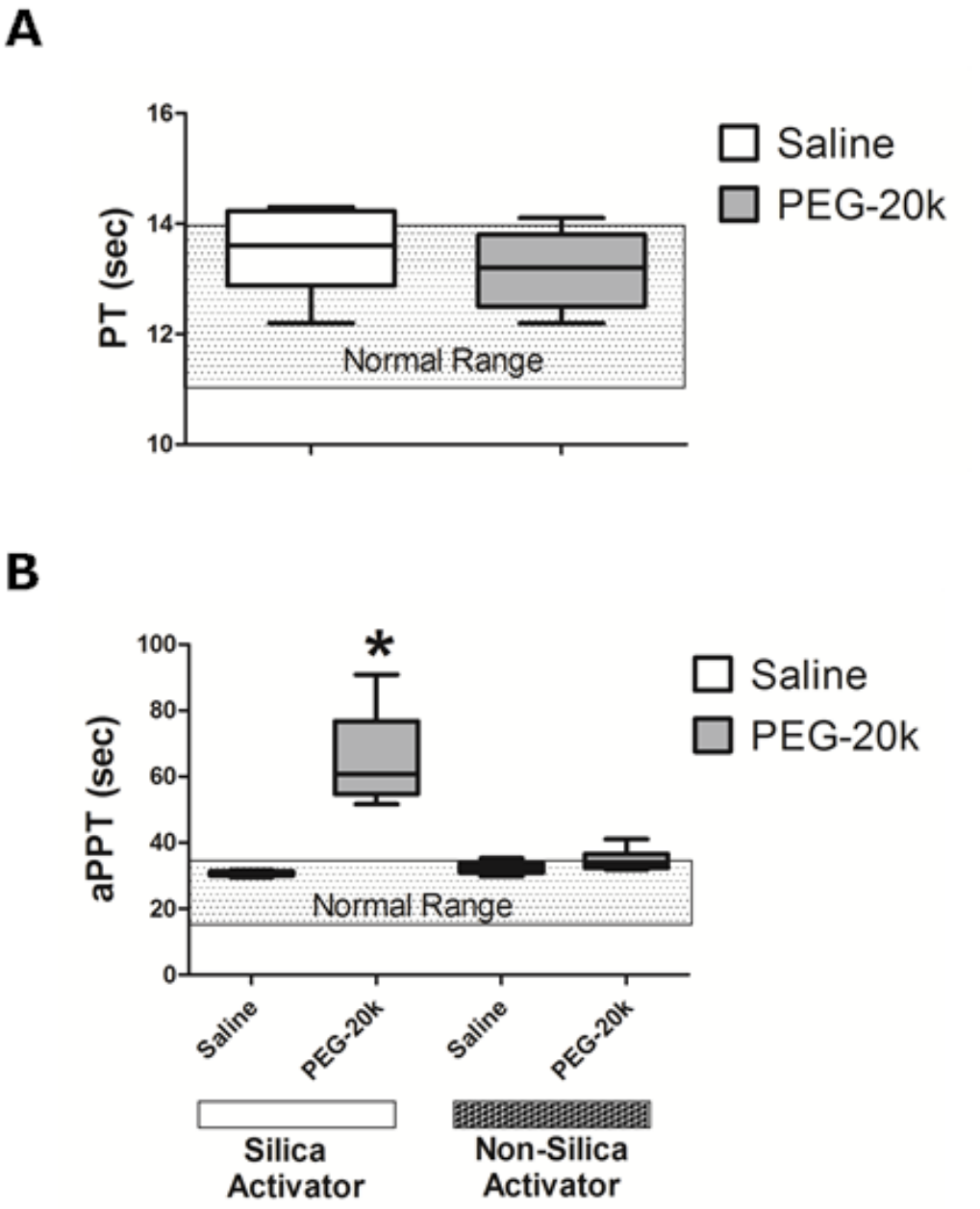
Saline
PEG-20k

Figure 2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14
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Normal Range
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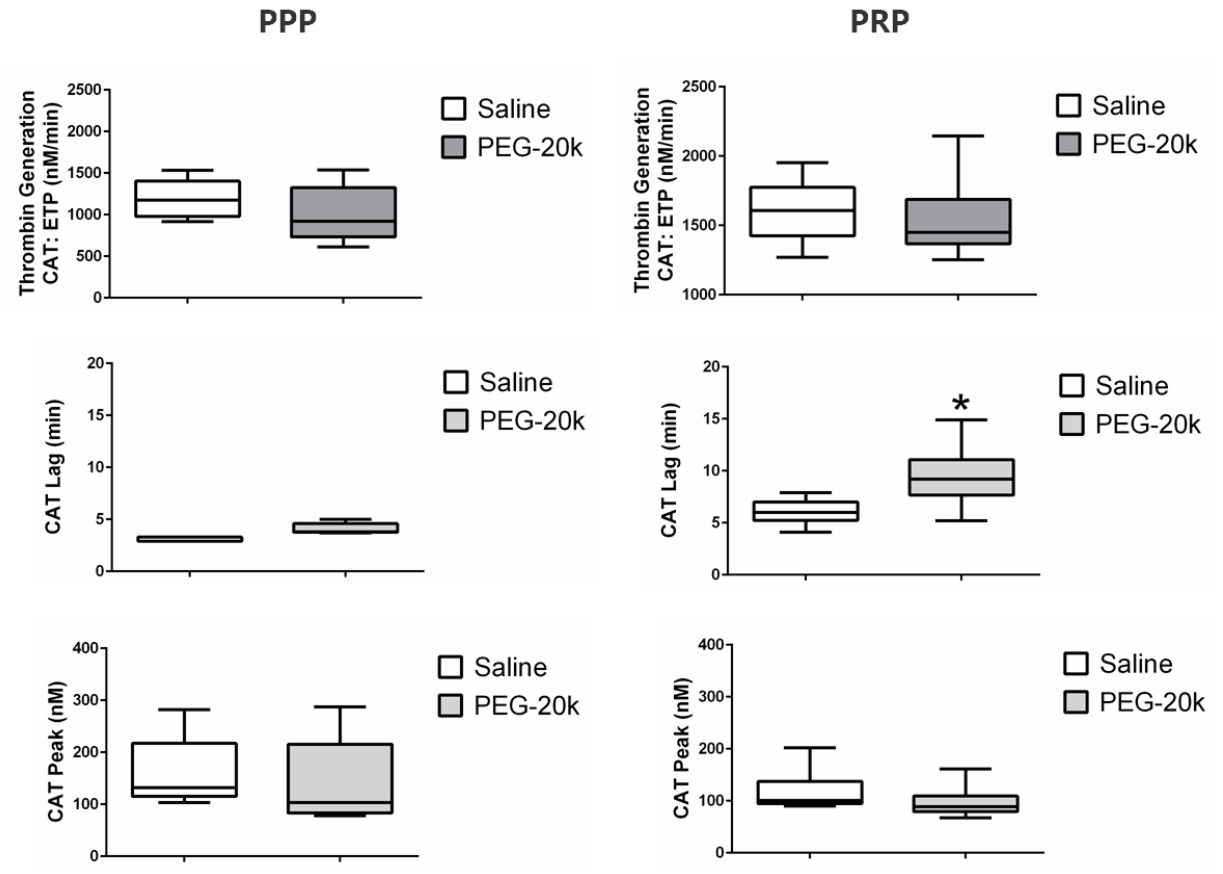
PEG-20k
Silica Activator
Non-Silica Activator

Figure 3


PPP
PRP
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CAT Peak (nM)
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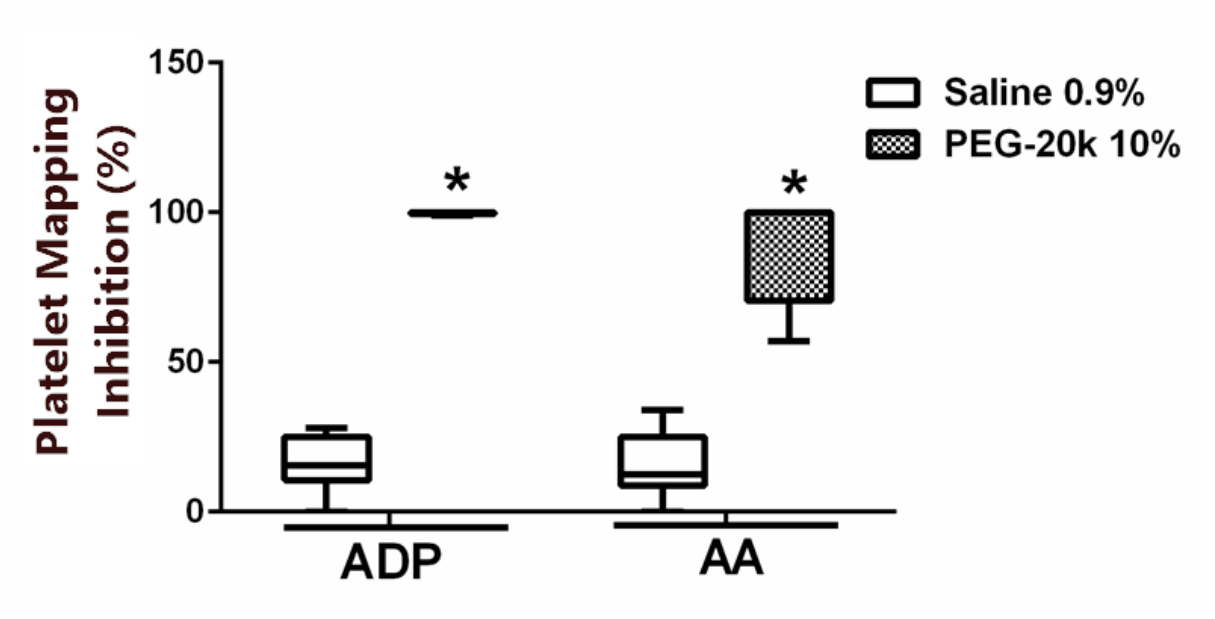
Figure 4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ADP
AA
Saline 0.9%
PEG-20k 10%

Figure 5

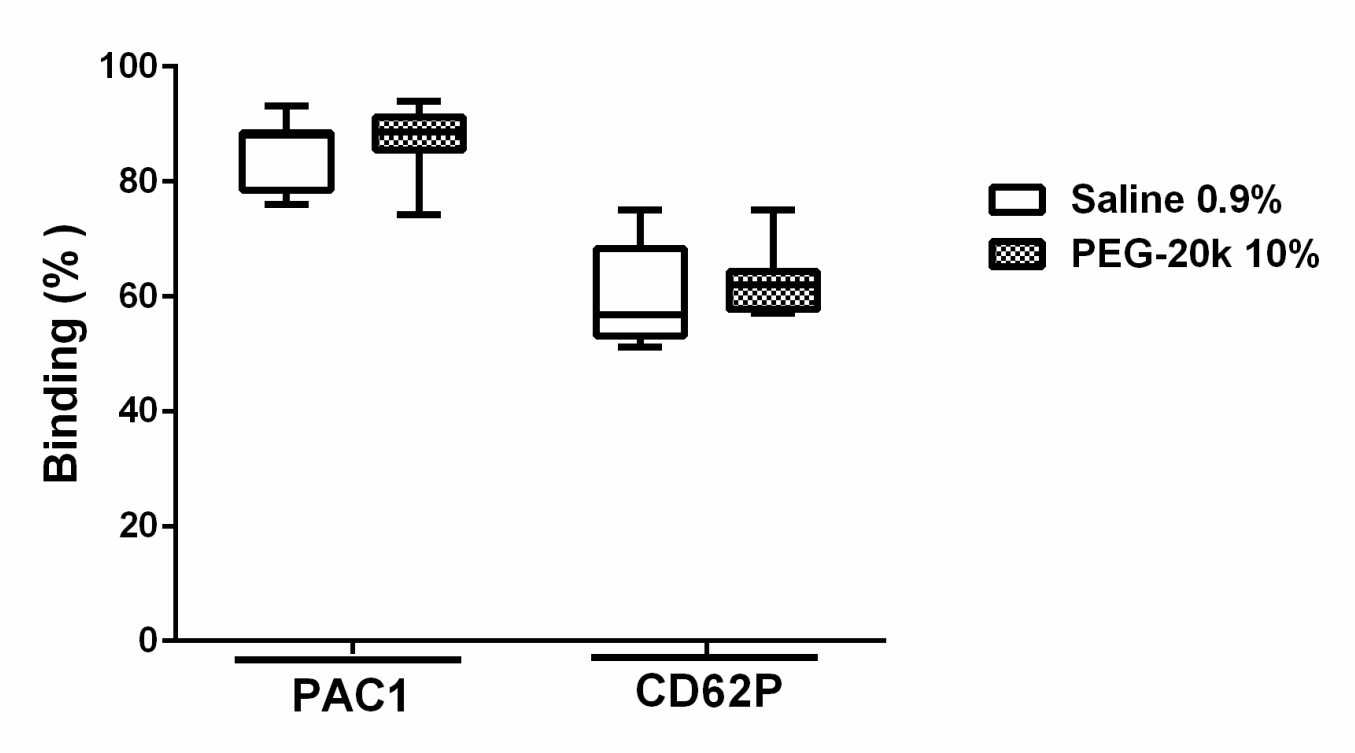
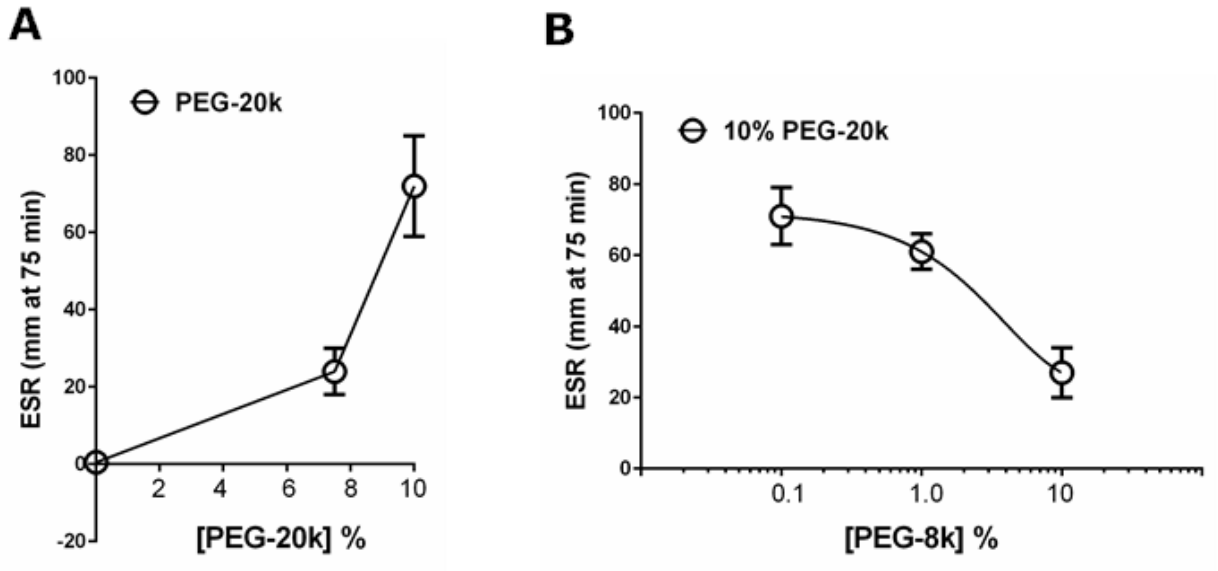


Figure 6


A
PEG-20k
ESR (mm at 75 min)
100
80
60
40
20
0
-20
2
4
6
8
10
[PEG-20k] %
B
10% PEG-20k
ESR (mm at 75 min)
100
80
60
40
20
0
0.1
1.0
10
[PEG-8k] %