Hemostasis and Coagulation Following Uncontrolled Hemorrhage and Resuscitation with Polymerized Hemoglobin Based Oxygen Carrier (HBOC-201) in Swine

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ABSTRACT

INTRODUCTION: *Coagulopathy are common complications of hemorrhagic shock (HS). Recently HBOC-201, a novel fluid with oxygen carrying capacity, has been proposed for hypotensive resuscitation and stabilization of HS patients. Coagulation and hemostasis have been studied in a swine uncontrolled hemorrhage model comparing HBOC-201 to standard resuscitation fluids.* ***METHODS***: *Yucatan mini-pigs (n=24) underwent uncontrolled hemorrhage by laceration and crush injury of a liver lobe. These animals were either non-resuscitated or resuscitated with HBOC-201 or buffered hydroxyethyl starch (HEX) during the 4 hr period following HS, after which they received full hospital care up to 72 hr. In addition to in-vivo parameters (blood loss and in vivo bleeding time (BT)), changes in hemostasis were evaluated by laboratory assays (coagulation (PT, PTT, fibrinogen), thromboelastography (TEG), and closure (in vitro bleeding) time (PFA-CT). Lung histopathology was evaluated for evidence of adverse microvascular thrombogenic pathologic change* ***RESULTS***: *Hemodynamic parameters were restored more effectively in HBOC-201-resuscitated pigs than in HEX- or non-resuscitated pigs. BT and blood loss were not different in these groups,*

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although the fluid requirement for the 4 hour resuscitation was significantly reduced in the HBOC-201 group as compared to HEX (respectively 23 vs 12 ml/kg/min). HBOC-201 did not significantly change platelet function or coagulation parameters in the 4hr following trauma. HEX resuscitation did, however, reduce the maximum amplitude on TEG, indicating platelet inhibition. PFA-CT was elevated in both resuscitated groups. Over the 4 hours following the induction of uncontrolled hemorrhage, PT remained stable and PTT decreased by 25% in both treated groups. After 24 hours, most hematological parameters returned to normal, with the exception of PTT and fibrinogen; PTT remained mildly prolonged and fibrinogen severely in both HBOC-201 and HEX groups. In post mortem histopathologic examination of lung sections, alveolar edema and fibrin deposition were rare in all groups. None of the sections showed evidence of platelets mixed with fibrin or other signs of thrombi or disseminated intravascular coagulation. ***CONCLUSIONS****: Evidence of clinically significant coagulopathic effects of HBOC-201 was not found. The absence of such effects makes HBOC-201 an adequate "bridging" resuscitation fluid during evacuation of HS casualties without exacerbation of coagulopathy.*

1.0 INTRODUCTION

Coagulopathy develops in patients with hemorrhagic shock [1,2]. Eventually, in severe hemorrhage, coagulation factors in both extrinsic and intrinsic systems are exhausted. Under these circumstances, mortality and morbidity are substantially increased and abnormal coagulation indices (PT above 14 sec and PTT above 35 sec) are commonly observed [3]. It has been reported that early severe coagulopathy correlates with mortality using both Glascow Coma Scale and Injury Severity Score (GCS and ISS) [4,5]. Coagulopathy commonly occurs soon after injury and subsequently, hypovolemia results in progressive splanchnic hypoperfusion, transient hypercoagulability and ultimately hypocoagulability. These changes are due to accelerated depletion of platelets, as well as excessive consumption of fibrinogen and intrinsic coagulation factors. As hepatic dysfunction becomes prominent, the ability to synthesize fibrinogen and intrinsic coagulation factors is reduced while consumption is maximal [6]. These changes, in the presence of hypoperfusion, will lead to disseminated intravascular coagulation (DIC), triggered by the diffuse vascular and tissue bed injury. Multiple organ system failure (MSOF) ensues.[3]. Early transfusions of whole blood, platelets, fresh frozen plasma and cryoprecipitate, used in trauma management practice, have been life-saving in anticipation of surgical control of hemorrhage. Deployment of blood products in military *prehospital* environments is rarely feasible. Indeed, on the battlefield, or at the civilian site of events inducing hemorrhagic shock, the administration of resuscitation fluids (saline or hydroxy-ethyl starch (HEX)) provides immediate restoration of intravascular volume and provisional vital organ perfusion. However, these common resuscitative practices also cause hemodilution, and aggravate anemia, thereby diminishing per volume O_2 carrying capacity, and enhancing hypocoagulability, with dilution of platelets and circulating coagulation factors [7]. This is especially evident where evacuation of traumatic casualties is delayed, as is often the case in military actions [8]. In these circumstances, the ability to use a lower volume resuscitation strategy and a fluid with oxygen carrying capacity will protect intrinsic coagulation mechanisms, and protect vital organs against damage.

HBOC-201, a bovine polymerized hemoglobin with oxygen carrying capacity (Biopure Corporation, Cambridge, MA) has been shown to increase survival rates in animal models of controlled and uncontrolled hemorrhage [9]; experiments in our laboratory have confirmed this finding [10,11]. Bovine hemoglobin oxygen carriers have been reported to increase bleeding [19], in contrast to human *O*-raffinose crossed linked hemoglobin in which decreased bleeding was demonstrated. [12]. Studies in our laboratory are evaluating extensively the efficacy and safety of HBOC-201 as the preferred resuscitation fluid for trauma casualties. As part of this effort, the comprehensive assessment of the hemostatic and coagulation effects of HBOC-201 and HEX in uncontrolled hemorrhage are ongoing. In order to mimic battlefield casualties, we have developed a

swine model of abdominal penetration, liver crush and laceration. Our results are reported here with special attention to recovery of coagulation abnormalities following resuscitation.

2.0 MATERIAL AND METHODS

2.1 Animal hemorrhagic shock

2.1.1 Model

Twenty-four (24) anesthetized, intubated Yucatan mini-pigs (23.0 ± 8.5 kg) were used in a HS model simulating uncontrolled bleeding for the battlefield. The icrush and laceration of a liver lobe through an abdominal wound, resulted in a bleeding rate of 1.80 ml/kg/minute within the first 30 min from the damaged organ.

Time 0 designated initiation of the liver lobe crush and laceration. A *prehospital phase* simulated an "evacuation delay" period during which blood transfusions and surgical stabilization are not available, in common in military operations. Animals were not immediately resuscitated after injury. After 15 minutes (T 15) they were infused with 10 ml/kg of resuscitation fluid at over 10 minutes. Subsequent 5 ml/kg infusions were administered at 30, 60, 120 and 180 minutes, if prospectively defined criteria were met (i.e., mean arterial pressure (MAP) < 60 mm Hg or heart rate (HR) increased in any value above baseline. Animals were intensively monitored for a total of 4 hours, but received only respiratory support and fluid resuscitation. After this 4 hour *prehospital phase*, *hospital care* was simulated by surgical repair of the liver and access to blood transfusion. Animals received either crossmatched banked whole blood transfusions or normal saline at 4, 24, and 48 hours, for a prospectively defined threshold value of Hb < 7 g/dL at each of these hospital care phase time points. All swine were euthanized at 72 hours.

2.1.2 Fluid resuscitation

Swine were randomly allocated to one of three resuscitation study groups (n=8 in each): No-resuscitation (NON), HBOC-201- and HEX- resuscitation. HBOC-201 is purified and ultrafiltered bovine stroma free Hb that is heat-treated and gluteraldehyde-crosslinked to form polymers with MW up to 500KD, prepared in a 50:50 racemic D and Lactated Ringers (LR) solution [13]. HEX, the standard fluid for battlefield resuscitation presently employed [8], is a 6% hydroxy-ethyl starch (MW=670Kd) prepared in balanced LR solution (Hextend, Abbott Laboratories, Abbott Park, IL).

In vivo monitoring: Vital signs and physiologic monitoring were performed as described elsewhere [10]. Bleeding time measurements were taken at Time 0 and 4 hours after injury. Bleeding time was performed by an ear incision with a scalpel blade # 11 on the ear edge to create a reproducible 5 mm anterior incision. The time for the bleeding to stop was recorded by the paper blotting method (Whatman paper #1).

2.2 In vitro monitoring

2.2.1 Blood Collection

Blood specimens were collected in vacutainer tubes at Times 0, 30, and 60 min, as well as at 3 and 4 hours after injury. Animals were maintained under anesthesia for 4 hours, and subsequently were recovered and extubated. Blood samples were obtained at 24, 48, and 72 hours during the *hospital* phase. (BD vacutainer, Palo Alto, CA).

2.2.2 Assays. Laboratory studies included: complete blood count (CBC) (Pentra 60C+ cell counter, ABX Diagnostics, Irvine, CA), thromboelastography (TEG), ADP-collagen capillary closure time (PFA-CT),

standard coagulation parameters (prothrombin time (PT), partial thromboplastin time (PTT), thrombin time (TT), anti-thrombin (ATIII), and fibrinogen.

2.2.2.1 TEG [14,15]: Thromboelastography (TEG) evaluates clot formation dynamics (TEG, Haemostasis Analyzer, Haemoscope Corp, Skokie, IL). 20 µl of 0.25 mM $CaCl_2$ and 340 µl of whole blood were pipetted into an oscillating cup. A pin connected to a torsion wire transmits the motion signal generated by clot retraction; this is integrated into a digitally based score. The reaction time (TEG-R) corresponds with initiation of fibrin formation and depends mainly on plasma factors. TEG-K and TEG-α are measurements of the kinetics of clot formation and reflect platelet adhesion on newly formed fibrin and rate of fibrin polymerization, respectively. TEG-MA measures maximal clot strength and shear modulus, and is dependent on platelet number and function, as well as plasma proteins to a lesser extent [14]. TEG-Ly (done at T 30 minutes) measures fibrinolyis due to tissue plasminogen activator (t-PA) activity, and is indicative of the presence of fibrin degradation products (FDP). Computed indices such as TEG-G (the clot firmness) and CI (the coagulation index were also reported. CI was defined as CI = 0.0184 * TEG-K + 0.1655 * TEG-MA – 0.0241 * TEG-α - 0.2454 * TEG-R - 5.022. [15].TEG-G was derived as TEG-G= 5000*TEG-MA/(100-TEG-MA),

2.2.2.2 PFA-CT: The platelet function analyzer (PFA-100, Dade Behring, Fl) measures capillary *closure time* [CT] and corresponds to *in vitro* bleeding time [16]. 800 µ of whole blood is vacuum aspirated through a 100 µm diameter capillary membrane coated with collagen and adenosine diphosphate (ADP). This promotes platelet adhesion and aggregation. Once a platelet plug has formed, blood flow ceases. This time to aperture occlusion by the platelet plug is referred to as the *closure time* (CT). CT is increased by low hematocrit, low platelet count, and low von Willbrand Factor (vWF) levels; it is unaffected by coagulation factor deficiencies and hypofibrinogenemia [16]. Thus CT is uniquely suited to measure coagulability in traumatic uncontrolled hemorrhage.

2.2.2.3 Coagulation assays: Coagulation assays were performed on a Stat Compact (Diagnostica Stago, Parsippany, NJ). This is a fully automated instrument using both mechanical magnetic ball (PT, PTT, TT, and fibrinogen) and colorimetric principles (ATIII).

2.2.2.4 *Histology:* Detection of microthrombi and fibrin deposition was performed by electron microscopy (EM) on lung sections obtained at necropsy. Sections were processed as previously described . Briefly, lungs were fixed in 4F1G fixative (4% paraformaldehyde, 1% gluteraldehyde), post-fixed in 2% osmium tetroxide, dehydrated in graded alcohols and Epon-embedded. Thick block sections were examined by light microscopy, and thin sections were stained with lead citrate and uranyl acetate, and examined in an LEO 912 AB electron microscope.

2.2.2.5 Statistics: Results, data and figures, are presented as means, and standard deviation or as otherwise stated. Data were analysed with a two-tailed paired Student's t-test, or equal variance Student's t-test. Statistical significance was considered for $p < 0.05$.

3.0 RESULTS

3.1 In Vivo

3.1.1 Physiology [10]: MAP at baseline was comparable in all three groups and significantly decreased from 69.6±3.2 to 27.6±2.9 mmHg after hemorrhage ($p<0.01$) at T 30 min. MAP was restored more rapidly

with HBOC-201 resuscitation at 30 minutes than with HEX. In the HBOC-201 group, 7 animals (87.5%) survived compared with only 1 (12.5%) with HEX and NON resuscitation ($p<0.01$ (Fisher exact)). The 15 animals that did not survive, died between 30 and 300 min after onset of hemorrhage.

3.1.2 Fluid requirement [20]: All animals in each group received resuscitation fluid infusion at 10 ml/kg at T 15 minutes. Using the vital sign criteria described above, all required second (5 ml/kg) infusions as well. At 60 minutes after injury, there was no significant difference between the required infusion volume in HBOC-201 or HEX resuscitated animals (17.0±4.0 and 19.6±2.0 ml/kg respectively). Since a large proportion of the HEX resuscitated animals died during the *prehospital* phase, the required fluid volume infusion was normalized to the survival time in order to enable comparison at later time points. After T 60 min, animals in the HBOC-201 group received significantly less fluid compared to HEX, (12±4 vs 23±14 ml/kg/min, respectively). At 4 hr(simulated "hospital arrival") none (0/8) of HBOC-201- animals received blood transfusion whereas 100% (3/3) of HEX-resuscitated pigs were transfused on the basis of Hb < 7 gm/dl.

3.1.3 Blood loss [20]: At 60 min post-resuscitation blood loss in this model was not significantly different among the three groups. At 4 hr blood loss was 49±12 and 58±10 ml/kg ($p>0.05$) for HBOC-201, and HEX groups, respectively.

3.1.4 In vivo bleeding time (BT): BT was measured at 0 and 4 hours post-hemorrhage. Only one non-resuscitated pig survived at 4 hr and BT was elevated compared to time 0. There were no statistically significant differences in BT values found between 0 and 4hrs in the fluid-resuscitated groups. Neither were there any detectable differences between the HEX and HBOC-201 groups at each time point ($p>0.05$) (Figure 1).

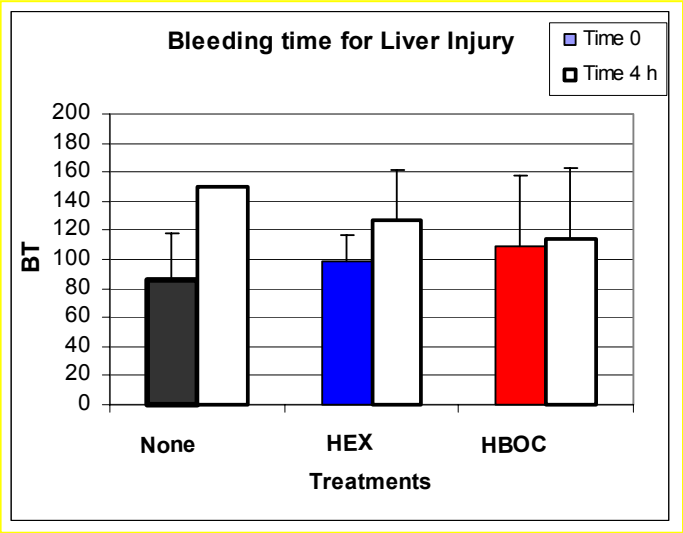

Figure 1: *In vivo* bleeding time (BT) at Times 0 (filled bar) and 4 hours (open bars), in non- (NONE) and HBOC-201-, HEX- (hetastarch) resuscitated animals. Mean and standard deviation

3.2 In vitro results

3.2.1 Hematology: At Time 4 hours, hematocrit (Hct) decreased similarly by ~14% from time zero(~29%) in both fluid-resuscitated groups, likely due to relative hemodilution. This is in contrast with NON animals, where Hct increased by 7%. As expected, hemoglobin concentration (Hb) paralleled the Hct in HEX- and

NON-pigs, but was higher in HBOC-201- than HEX-pigs due to the delivery of hemoglobin by HBOC-201. The mean hemoglobin load in HBOC-201 infusions was 3.1±0.8 g/kg, resulting in a mean peak plasma concentration of 5.3±1.0 g/dl at 4 hours. Platelets decreased similarly in the two treatment groups, from ~420 x 10^6/ml at Time 0 to less than 180 x 10^6/ml at Time 4 hours, probably from hemodilution. We noticed a trend toward a similar platelet reduction in the non resuscitated group at 60 min and 3 hr, probably reflecting consumption.

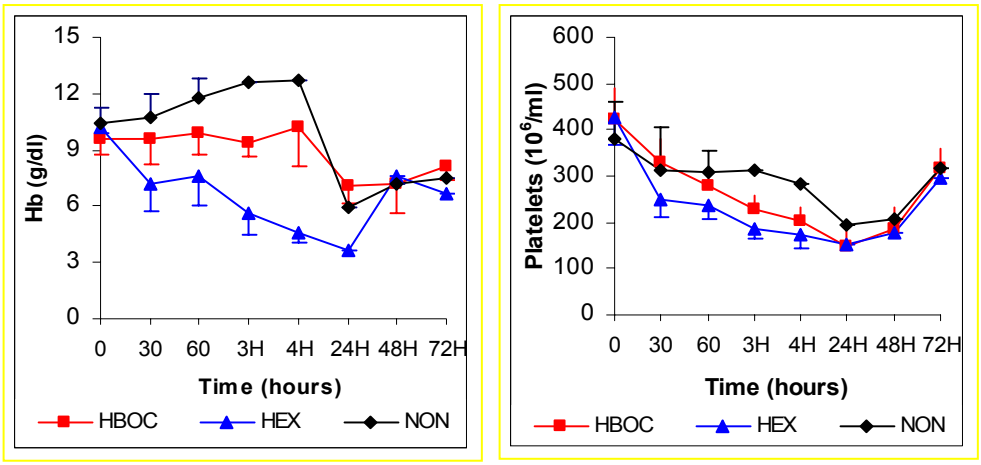

Figure 2: Hematology data for the 3 groups studied: HBOC-201 (square), HEX (triangle) and NON (diamond). Hemoglobin concentration (Hb) and platelet concentration. Note the hemoglobin hemoconcentration during the *prehospit*al phase for NON, hemodilution for HEX and compensation by HBOC-201addition for HBOC. Mean and standard deviations are shown.

3.2.2. Ex vivo bleeding time: PFA-CT decreased slowly over the first three hours in the NON group and gradually increased back to time 0 values between 4 and 24h (Figure 3). Both HEX and HBOC-201groups showed a marked increased in closure time between 3h and 24 h. Although not statistically significant in this sample, the data suggest a difference between HBOC-201 and HEX groups at 24 hr. HBOC-201 may have a longer lasting effect on PFA-CT than HEX.

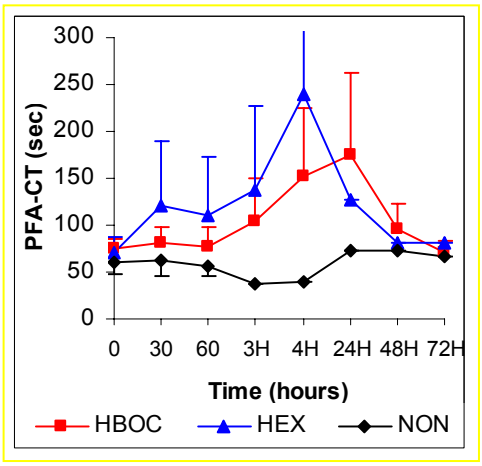

Figure 3: PFA-CT for the 3 groups studied: HBOC-201 (square), HEX (triangle) and NON (diamond). In vitro bleeding time as evaluated by the closure time (PFA-CT) in an ADP/collagen coated capillary increased for HEX and HBOC-201 compared to the NON. Mean and standard deviation ranges shown.

3.2.3 Thromboeleastography: Neither TEG-R, nor TEG-MA for non resuscitated animals did change with time in the *prehospital* phase, as shown in the Figure 4 graphs. TEG-R also did not change for the treated group in the *prehospital* phase. TEG-K, TEG-alpha indicated no difference compared with time 0 (data not shown). After 24 hr, the HBOC-201 group showed a significant change ($p<0.01$), characterized by an increase in reaction time. This was difficult to compare to the HEX group, as only one animal survived to this timed data point. At 4 hrs, TEG-MA was significantly higher in NON than in HBOC-201. As well, HBOC-201 was higher than in HEX.

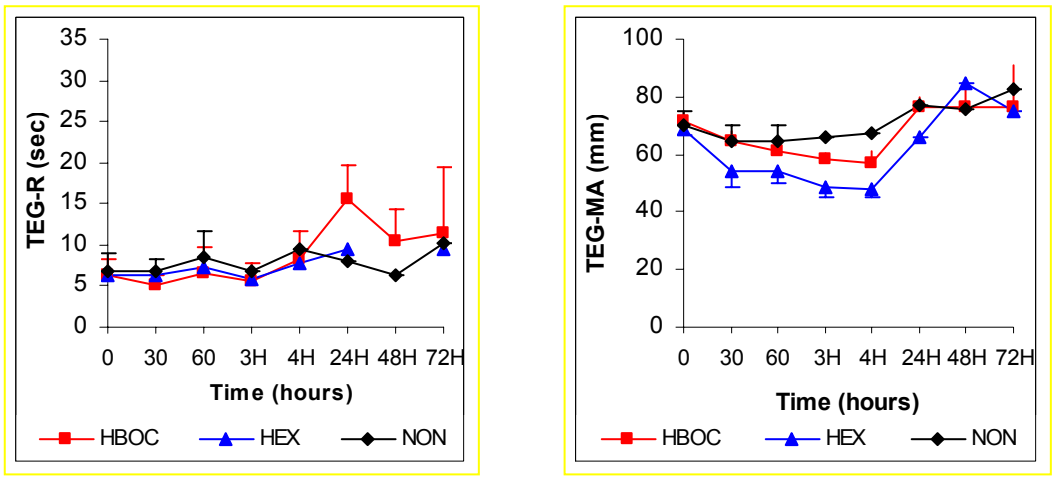

Figure 4: Thromboelastography for the 3 groups studied: HBOC-201 (square), HEX (triangle) and NON (diamond). Measure of the reaction time (TEG-R) and the maximum amplitude (TEG-MA). Mean and standard deviation are shown.

3.2.4 Coagulation parameters: PT remained stable in NON and HBOC-201 groups after injury. However, in HEX treated animals, PT increased steadily, and was significantly different ($p<0.01$) at 4h. PTT decreased slightly in the treated animals compared with NON, and remained stable for 4 hr; this difference did not reach statistical significance. After 24 hours PTT remains elevated in all groups, probably indicative of the presence of intrinsically produced heparin, unopposed by hepatic coagulation factors.

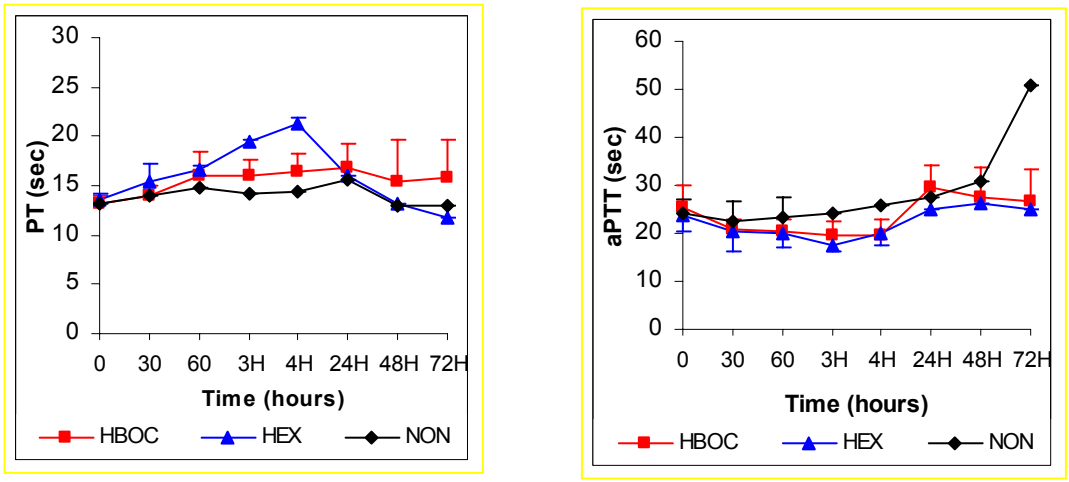

Figure 5: Measure of coagulation indices (PT and PTT) for all the 3 groups studied: HBOC-201 (square), HEX (triangle) and NON (diamond). Mean and standard deviation

3.2.5 Histopathological lung sections: Electron micrographs of lung tissues taken at day 3 showed modest intraalveolar fibrin deposition and edema in 62% of HBOC-201, and 37% of HEX treated animals. This difference was not statistically significant, and may be caused by the fact that HEX treated animals died at earlier time points. No platelet aggregates or microthrombi were found in pulmonary blood vessels.

4.0 DISCUSSION

The coagulation status in patients with uncontrolled hemorrhage is difficult to evaluate because fluid administration can significantly affect the monitored parameters [2]. The experimental model presented here allows us to study the coagulation profile under 3 different conditions up to 3 days with and without resuscitation. Hemoconcentration and hemodilution strongly influence coagulation indices and distinguish the groups presented here. Obviously, HS induces consumptive coagulation; as well, plasma factors are further reduced during HBOC-201 or HEX resuscitation. As expected, hemoconcentration occurred during hemorrhage in the NON animals as evidenced by the increase in Hct. In contrast, hemodilution develops after both HBOC-201- and HEX- resuscitation, and changes is Hct and cell count confirm this. The decline of total platelets as seen in the NON group indicates a consumptive mechanism, The contribution of platelets to wound stasis in this liver injury model, is demonstrated by a small decrease in platelet activation (PFA-CT). PFA-CT (as well as BT) is known to be prolonged by a number of factors. These include reduction of platelet number, decreased levels of vWF factor, and low hematocrit. The remarkable increase of PFA-CT after fluid resuscitation, is likely due to the hemodilution by HBOC-201 or HEX between 20 min and 3 hrs, most likely related to the relative thrombocytopenic state induced by dilution with both resuscitation fluids. This effect becomes fully apparent at 4h, and suggests that there may be a delayed action of both fluids on platelet function. However, there is an important difference between HBOC-201 and HEX; PFA-CT and TEG-MA are both lower than with HEX (also seen in the controlled HS model) [11] suggesting that HBOC-201 may stimulate platelet function more than HEX. The elevated PT at 4 h underlines the stronger coagulopathic effect of HEX compared to HBOC-201, probably due to hepatic dysfunction. The origin of this difference is not clear in this data set, and awaits further study. Intrinsic coagulation pathway activity, as reflected by PTT decline, in both resuscitation fluid groups is indicative of the relative physiologic importance of the instrinsic system in this model.. The coagulopathic effect of HBOC-201 seems to be most evident at 24 h as TEG-R and PT remain elevated. All these effects are mild and transient, resolving by 72 hours.

HS induces hypercoagulation which is followed by hypocoagulation after fluid resuscitation. The change in the indices after HEX infusion is comparable to the data presented by Ledgerwood et al [2].Recent reports of NO scavenging activity in HBOC-21 experiments [18] might imply that platelet activation would be expected. The absence of microvascular thrombosis noted at necropsy do not support this hypothesis and suggests that NO scavenging is not a significant procoagulant force in vivo.[19]. Lee et al. [12], using *O*-raffinose as hemoglobin based carrier, showed shortened BT and enhanced clot formation, whereas we did not see significant changes in BT although we observed a significant hypocoagulation. Lee's model is significantly different than ours, and this may explain the conflicting results.

5.0 CONCLUSIONS

In summary, TEG data from the controlled hemorrhage model suggest that HBOC-201-resuscitation may induce a mild change in clot dynamics particularly at 24 hr after injury in this model of uncontrolled hemorrhage. This phase is transient and the indices returned to baseline at 72 hr. These data suggest that HBOC-201 could be an adequate "bridging" resuscitation fluid during evacuation to hospital, and that the effects of HBOC-201- on hemostasis do not impair survival, and are unlikely to be clinically significant.

6.0 ACKNOWLEGDEMENTS

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The opinions contained herein are the ones of the authors and are not to be construed as official or reflecting the views of the Navy department, or Department of Defense, or the U.S. Government.

The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. The study was approved by the WRAIR/NMRC Institutional Animal Care and Use Committee (IACUC) and all procedures were performed in an animal facility approved by the American Association for Accreditation for Laboratory Animal Care (AALAC).

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