Bovine Polymerized Hemoglobin (HBOC-201) for the Prehospital Resuscitation of Combat Casualties with Hemorrhagic Shock (HS)

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ABSTRACT

OBJECTIVES: To compare the physiologic effects of BPH vs. hetastarch (HEX), the current resuscitative fluid used by U.S. Special Forces, in delayed resuscitation HS models simulating battlefield injuries. METHODS: After induction of HS in controlled (catheter withdrawal) and uncontrolled (liver injury) hemorrhage swine models, the effects of BPH, HEX, and no resuscitation (NON), followed by hospital-like care after a 4 hour "evacuation delay", were compared. Standard physiologic parameters were followed for 72 hours. Hemostasis was evaluated by routine coagulation assays, thromboelastography, collagen/ADP-coated membrane aperture closing time, and platelet aggregation ADP-release. Leukocyte adhesion and immunophenotype were compared using FACS. Plasma cytokines were assayed by ELISA and Western Blot. RESULTS: In controlled HS, 100% (8/8) of BPH, 88% (7/8) of HEX, and 63% (5/8) of NON pigs, survived to

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72 hours (p=0.04). MAP (p=0.01), SVRI (p=0.08), and MPAP (p=0.06) were higher in BPH pigs as compared to HEX. By 90 minutes, cardiac index reached baseline in the BPH group (4.2 ± 0.3 L/min/m2) but was 1.4-fold greater than baseline in the HEX group (6.7 ± 0.5 L/min/m2, p<0.001). BPH pigs had a lower fluid requirement (716 vs. 1226 ml, p=0.004) in the prehospital phase. 12% of BPH vs. 100% of HEX pigs required blood transfusions (p=0.002) in the hospital phase. Although transcutaneous tissue oxygenation (TCOM) levels were higher in BPH pigs (i.e., 34.6 ± 5.0 vs. 15.2 ± 3.0 mmHg at 90 minutes, p<0.001), lactate, urine output, and creatinine levels were similar (p=NS). In uncontrolled HS, 7/8 (87.5%) BPH, 1/8 (12.5%) HEX, and 1/8 (12.5%) NON pigs survived 72 hours (BPH vs. HEX p=0.01). BPH pigs had higher systemic and pulmonary artery pressures and had comparable cardiac outputs, but were less tachycardiac in comparison to HEX pigs. Baseline TCOM levels were restored more rapidly in BPH pigs (45 vs. 220 minutes) and lactic acidosis was less severe (3.2 ± 0.7 vs. 6.7 ± 4.4 at 60 minutes). Although BPH pigs received similar amounts of IV fluid, urine output was higher (2.3 ± 1.5 and 1.0 ± 1.1 ml/kg). HEX pigs had higher blood loss than BPH pigs (51.8 ± 3.2 and 43.2 ± 4.1 ml/kg respectively). In comparison with HTS, BPH had no significant effects on coagulation and platelet function parameters. In controlled HS, expression of PMN beta 2-integrins (CD11b, CD18), and the lymphocyte alpha 4-integrin (CD49d), were lower in BPH- than HEX-pigs (~2.7, 4.8, and 1.5-fold). Increased elaboration of the anti-inflammatory Th2 cytokine, IL-10, was seen in BPH pigs (~1.7-fold). The CD4/CD8 ratio and the plasma level of the pro-inflammatory cytokine, IL-2, were not significantly different. CONCLUSIONS: In comparison with HEX, BPH restored tissue oxygenation, decreased lactic acidosis, diminished proinflammatory neutrophil activation, and improved survival, without increased hemorrhage or deleterious effects on hemostasis, in controlled and uncontrolled HS models. Clinical trials should be completed to definitively compare the efficacy and safety of BPH and traditional asanguinous resuscitative fluids, for the resuscitation of HS casualties.

1.0 INTRODUCTION

Hemorrhage accounts for the preponderance of salvageable combat casualty mortality, especially with evacuation delay. After securing hemostasis, blood transfusion may be life-saving pending surgical stabilization, however, transfusion capability is costly, rarely supports echelons I/II units and USN ships, and the walking blood bank is impractical. Thus, a "bridging" volume replacement fluid with O_2 transporting properties is urgently needed to improve survival of hemorrhagic shock casualties aboard US Navy vessels and on the battlefield of the 21st century. A low volume hemoglobin substitute that is room temperature stable, has no blood typing and banking requirements (i.e., universal compatibility), is easy to administer, and is efficacious and safe as a "bridging" replacement fluid for early resuscitation of hemorrhagic shock (HS) combat casualties, should fill that current therapeutic hole [1]. Hemoglobin based oxygen carriers (HBOC) transport O_2 and provide colloid volume replacement (something in between crystalloid fluid and RBC transfusion) and thus, they are potentially ideal resuscitative fluids for hemorrhagic shock casualties in the field, aboard US Navy vessels, and for Special and other operations in which delayed blood transfusion and prolonged evacuation times are expected.

2.0 STUDY OBJECTIVE

To compare the efficacy and safety of HBOC-201 (bovine polymerised hemoglobin [BPH], Hemopure®, Biopure Corp. MA) with 6% hetastarch in balanced salt solution (HEX, Hextend®, Abbot Laboratories, IL) and no resuscitation in swine models of HS with controlled and uncontrolled hemorrhage incorporating a four hour delay to definitive medical treatment (Figure 1).

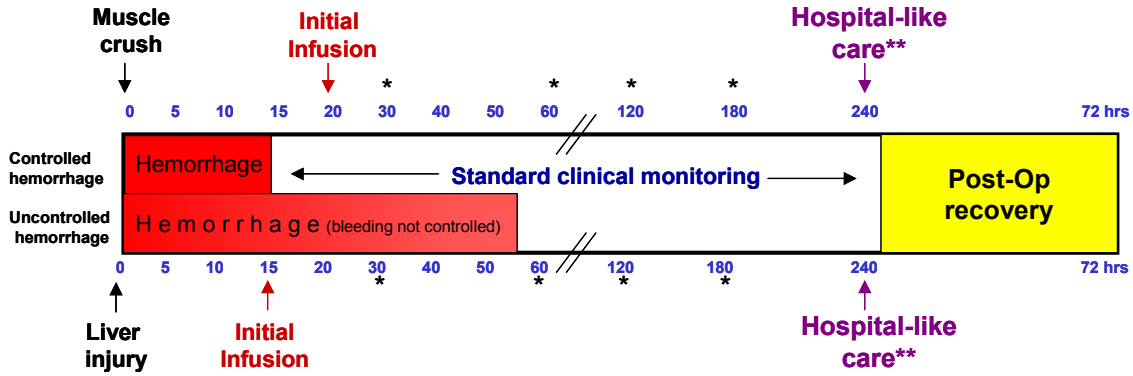


Figure 1: Experimental timeline

3.0 MATERIALS AND METHODS

3.1 Animal husbandry and preparation

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 NMRC Institutional Animal Care and Use Committee (IACUC) and all procedures were performed in an animal facility approved by the Association for Assessment and Accreditation for Laboratory Animal Care International (AAALAC). Swine were allowed to acclimate to the animal facility for 1 week with free access to feed and water. Feed and water were withheld 12-14 hours prior to initiation of the experiment to induce dehydration.

3.2 Injury, hemorrhage and resuscitation procedures

3.2.1 Anesthesia: Animals were sedated and anesthesia induced with intramuscular ketamine hydrochloride (33 mg/kg), atropine sulfate (0.05 mg/kg), and mask ventilation with isoflurane (3.0%) and 100% O_2 to facilitate endotracheal intubation. Anesthesia was maintained via isoflurane (1%-2.5%) in 21% O_2. Pigs were ventilated for anesthesia-induced apnea (Ohmeda 7800 series ventilator, Datex, Madison, WI) (12-15 breaths/minute; tidal volume 5-10 ml/kg; and FiO_2 0.21).

3.2.2 Instrumentation: The right external jugular vein and carotid artery were dissected and isolated. A 9 F introducer sheath was placed in the external jugular vein using Seldinger technique and a 7.5 F pulmonary artery catheter (PAC; Edwards Life Sciences, Irvine, CA,) was inserted for continuous hemodynamic and cardiac output (CO) monitoring. A 20G angiocath was placed in the carotid artery and mean arterial pressure (MAP) was continuously transduced. Urine was collected via bladder catheterization. All surgical procedures were performed under aseptic techniques.

3.2.3 Tissue Injury and Controlled Hemorrhage: A 3-5 cm lower abdominal incision was made and the left rectus abdominis muscle located. The rectus sheath was bluntly mobilized and a Kocher clamp placed on a standardized portion of the muscle in the center of the incision. The Kocher clamp was closed for 5 minutes to create a soft tissue injury and pigs were hemorrhaged 40% or 55% of estimated total blood volume (EBV) via the external jugular vein and/or the carotid artery to induce HS. All "shed" blood was collected in blood bags containing citrate phosphate dextrose (CLX, Medsep Corp., Covina, CA) for later re-infusion.

3.2.4 Liver Injury and Uncontrolled Hemorrhage: A standardized liver injury was created by placing a ring clamp over the left lower lobe, ~50% in width and ~0.75-2.0" from the apex, adjusting for relative size of the liver and weight of the pig. The clamp was closed and an 11 blade was used to lacerate the lobe from the top of the clamp through the remaining width. The liver injury denoted the start of the pre-hospital phase (Time 0). After 1 minute, the clamp was removed and the remaining tissue excised, resulting in ~25% lobectomy, consistent with a grade III liver injury. Bleeding was spontaneous, unhampered, removed via intraperitoneal suction, and quantified by weight.

3.2.5 Resuscitation: Pigs were randomly allocated to one of three treatment groups: Hemoglobin based oxygen carrier (HBOC, HBOC-201®, Biopure Corp., Cambridge, MA); 6% hetastarch in LR (HEX, Hextend®, Abbott Laboratories, Abbot Park, IL); or no fluids (NON). Five minutes following controlled hemorrhage or at 15 minutes into uncontrolled hemorrhage, resuscitated pigs were administered 10 ml/kg of HBOC or HEX over 10 minutes. Additional infusions of 5 ml/kg were provided at 30, 60, 120, and 180 minutes post-injury if hypotension (MAP < 60 mmHg) or tachycardia (HR > baseline value [Time 0]) were observed. Fluids were infused at room temperature.

3.3 Post-operative clinical observations and treatment

Hospital arrival was simulated at 4 hours. Animals were administered 10 ml/kg autologous shed blood or allogeneic packed red blood cells (PRBC) for anemia (Hb <7 g/dL) and/or 10-20 ml/kg normal saline (NS) for hypotension. 13 mg/kg cephazolin (antibiotic), and 0.01 mg/kg buprenorphine (analgesic), were administered. The PAC was removed and the jugular vein introducer was secured for postoperative blood sampling and fluid administration. The arterial and bladder catheters removed and surgical sites repaired as necessary. Surgical incisions were closed and surgical dressings applied. Animals were extubated and recovered from anesthesia. Vital signs and general status were assessed 24, 48, and 72 hours post-injury. Pigs received 10 ml/kg NS, autologous shed blood or PRBCs as needed for anemia or hypotension as well as antibiotics and analgesia. Pigs were euthanized 72 hours post-injury for necropsy and histological analysis.

3.4 Data Collection and Analysis

Standard invasive and noninvasive hemodynamic parameters were monitored for 240 minutes during the simulated pre-hospital phase. In the liver injury, blood loss was measured by weighing collection canisters at 5 and 15 minutes (pre-resuscitation), and 20, 30, 60, and 240 minutes (post-resuscitation). Sponge weight was included in total post-resuscitation blood loss. Transcutaneous tissue oxygenation (TCOM or $tcpO_2$) was noninvasively measured with a TCM4 Tina monitor (Radiometer, Copenhagen, Denmark) using four Clark type polarographic electrodes (data represent mean values) positioned bilaterally on the upper torso and on the inner thighs. Blood gases (ABG and MVBG) were measured with an automatic analyzer (ABL 705, Radiometer, Copenhagen, Denmark). Blood samples were collected for complete blood counts (CBC, Pentra 60 C+, ABX, France), chemistries (Vitros 250 Analyzer, Ortho), and coagulation assessments (PT, PTT, StatCompact Diagnostica Stago, Asniere, France). Thromboelastography (TEG) was used to evaluate clot formation dynamics (TEG, Haemostasis Analyzer, Haemoscope Corp, Skokie, IL). 20 µl of 0.25 mM CaCl2 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 [2]. 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 clot firmness (TEG-G) and the coagulation index (TEG-CI) are also reported. TEG-CI was defined as TEG 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). Leukocyte adhesion and immunophenotype were compared using FACS. Plasma cytokines were assayed by ELISA and Western Blot.

3.5 Statistics

Physiology data are expressed as mean $\pm$ standard error of the mean (SEM) for animals alive at time of measurement. Hematology data are presented as mean $\pm$ 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 $p< 0.05$.

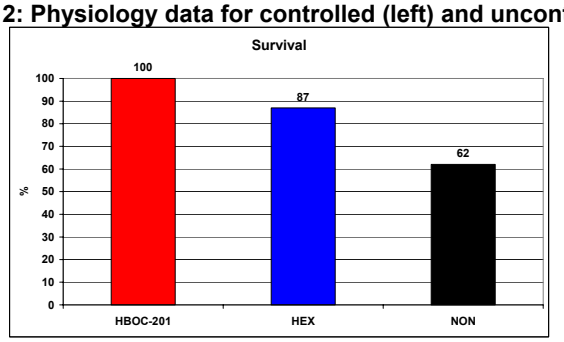
4.0 RESULTS

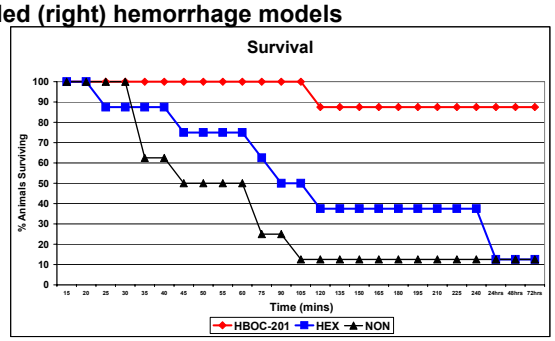
4.1 Physiology

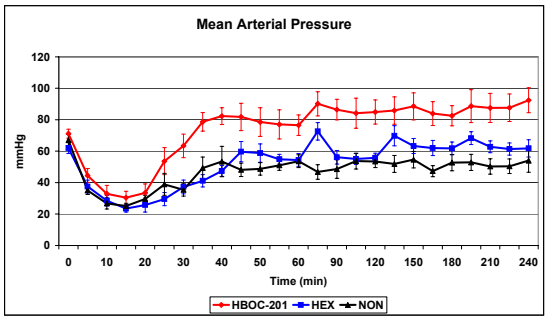
4.1.1 Tissue Injury with 40% EBV Controlled Hemorrhage: 100% (8/8) of HBOC-201-, 88% (7/8) of HEX-, and 63% (5/8) of NON-resuscitated pigs, survived to 72 hrs (Figure 2, left column). Mean arterial pressure (MAP), systemic vascular resistance (SVRI), and mean pulmonary arterial pressure (MPAP) were higher in the HBOC-201 group. By 90 minutes, cardiac index (CI) was restored to baseline in the HBOC-201 group and was 1.4-fold greater than baseline in the HEX-group following resuscitation. HBOC-201- treated pigs had a lower fluid requirement than HEX-treated pigs (716 vs. 1226 ml) in the pre-hospital phase and required fewer blood transfusions (12% vs. 100%) in the hospital phase. Urine output and creatinine levels were comparable in HBOC-201- and HEX- treated pigs. Tissue oxygenation levels (TCOM) were highest in the HBOC-201 group. In summary, except for decreased CI, the hemodynamics, lactic acid, base deficit, cutaneous tissue oxygenation, urine output, and survival were equivalent or better in HBOC-201-treated swine, despite lower IV fluid and blood transfusion requirements.

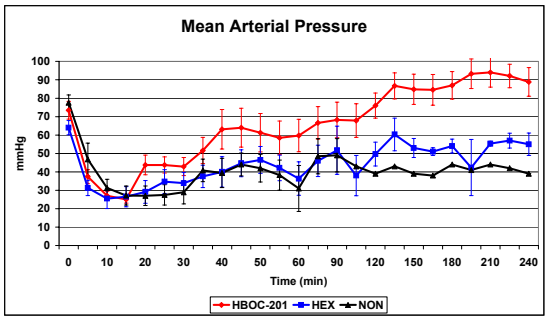
4.1.2 Liver Injury with Uncontrolled Hemorrhage: In order to simulate resuscitation in combat with severely delayed evacuation, NMRC evaluated HBOC-201 in a liver crush/laceration injury model (similar to Manning and Katz's model, [3], [4]) with extension of evacuation delay to 4 hours, spontaneous ventilation (FiO_2 0.21), and with accurate quantification of hemorrhage [5]. HBOC-201 was compared with HEX and no treatment (NON). Additional arterial catheter blood withdrawal was not done and hemorrhage into the abdominal cavity was measured. Resuscitation fluids were infused for MAP < 60 mm Hg and/or tachycardia. HBOC-201 stabilized hemodynamics, increased cutaneous tissue oxygenation, decreased base deficit and lactic acidosis, increased survival, decreased blood loss, and decreased fluid and blood transfusion requirements (Figure 2, right column).

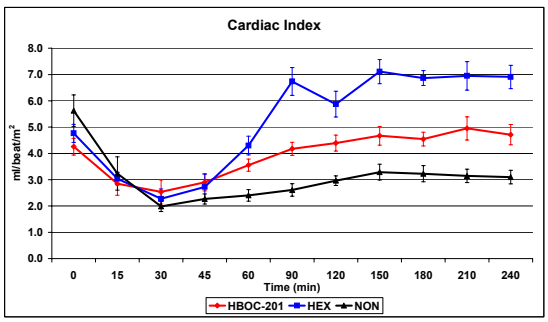
Figure 2: Physiology data for controlled (left) and uncontrolled (right) hemorrhage models

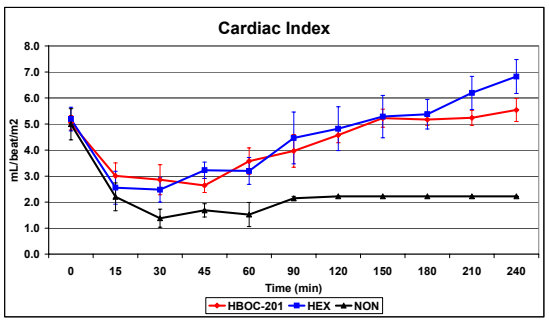


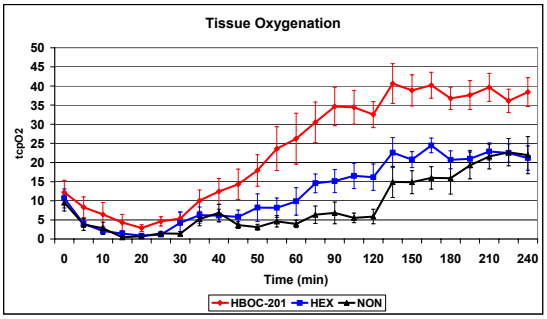


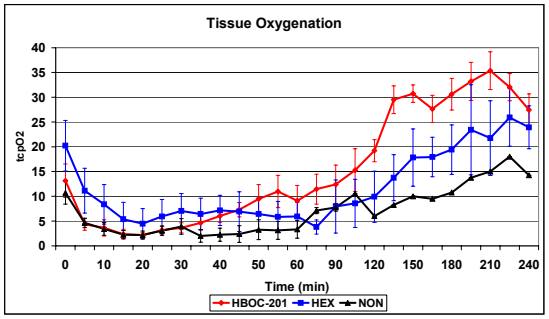


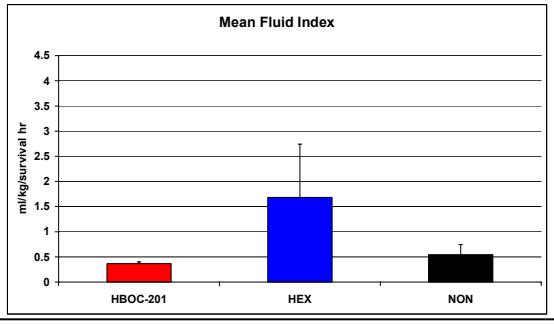


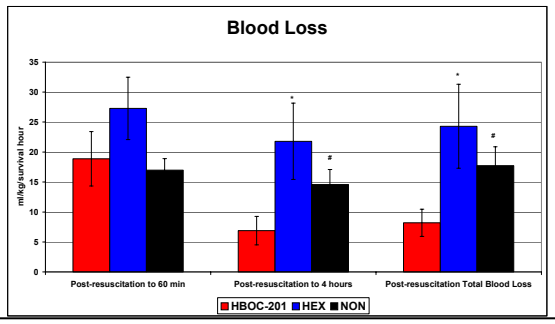












4.2 Hematology: Thromboelastography (TEG):

In both HS models (controlled and uncontrolled hemorrhage), the natural course of HS without resuscitation (NON-pigs) was characterized by an absence of significant changes in TEG-R, TEG-al, and TEG-MA (TEG-mean amplitude) (the former reflecting coagulopathic and latter reflecting thrombopathic alterations). In contrast, TEG-G and TEG-CI (clotting index) remained stable during the simulated prehospital phase but increased subsequently during the simulated hospital phase (Figure 3).

In the HS model with controlled hemorrhage, TEG-R began to increase in HBOC-201-pigs during the late prehospital phase and was greater than in NON- or HEX resuscitated pigs at 24 hours. A similar but mirror-image trend was seen for TEG-al. TEG-MA values were similar across treatment groups but a trend was appreciated with NON>HBOC-201>HEX. TEG-G increased during the hospital phase at 48-72 hours in HBOC-201-pigs. The TEG-CI appeared to be more affected in HBOC-201resusciated pigs, especially at 24 hours.

In the HS model with uncontrolled hemorrhage, as aforementioned, comparisons across treatment groups is hampered by the differential survival rates in the HBOC-201 and control groups. Nevertheless, possible trends were noted towards higher TEG-MA, TEG-G, and TEG-CI in HBOC-201- in comparison with HEX resuscitated pigs. In the HBOC-201 group, in which survival was high, despite lower fluid requirements (and thus less hemodilution), the overall pattern of TEG responses was similar to that noted in controlled hemorrhage. TEG-R increased and TEG-al decreased by 24 hours; TEG-MA decreased slightly initially and then stabilized; TEG-G and TEG-CI decreased during the pre-hospital phase and stabilized during the hospital phase (TEG-G exceeded baseline by 24 hours).

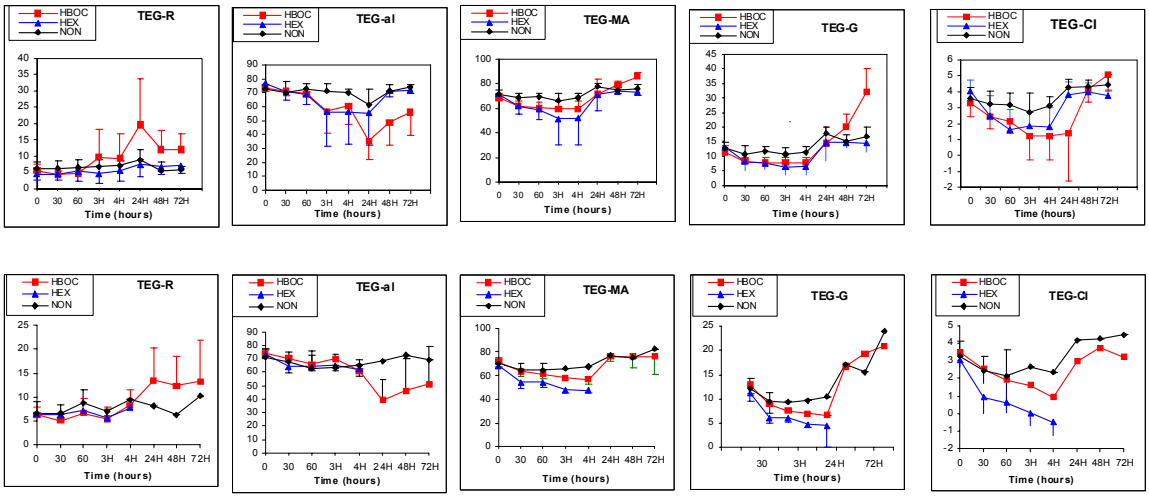


Figure 3. TEG in swine with HS with controlled (top) and uncontrolled hemorrhage (bottom)

4.3 Immunology: Leukocyte adhesion markers

In the swine HS model with controlled hemorrhage, the natural course (NON-treatment) of HS was shown be characterized by ~2-2.5-fold increased expression of PMN beta-2 integrins, CD11 and CD18 (Figure 4). Resuscitation with HEX increased beta-2 integrin expression even higher to ~3-5-fold the baseline value; in contrast, HBOC-201-treatment averted a significant rise in PMN beta-2 expression (p=0.001 and 0.01, respectively, at 4 hours). Lymphocyte alpha-4 integrin expression increased >1.5-fold in HEX-, but did not

increase in HBOC-201-treated pigs ($p>0.05$) (PMN alpha-4 integrin expression was not quantified in the controlled hemorrhage model).

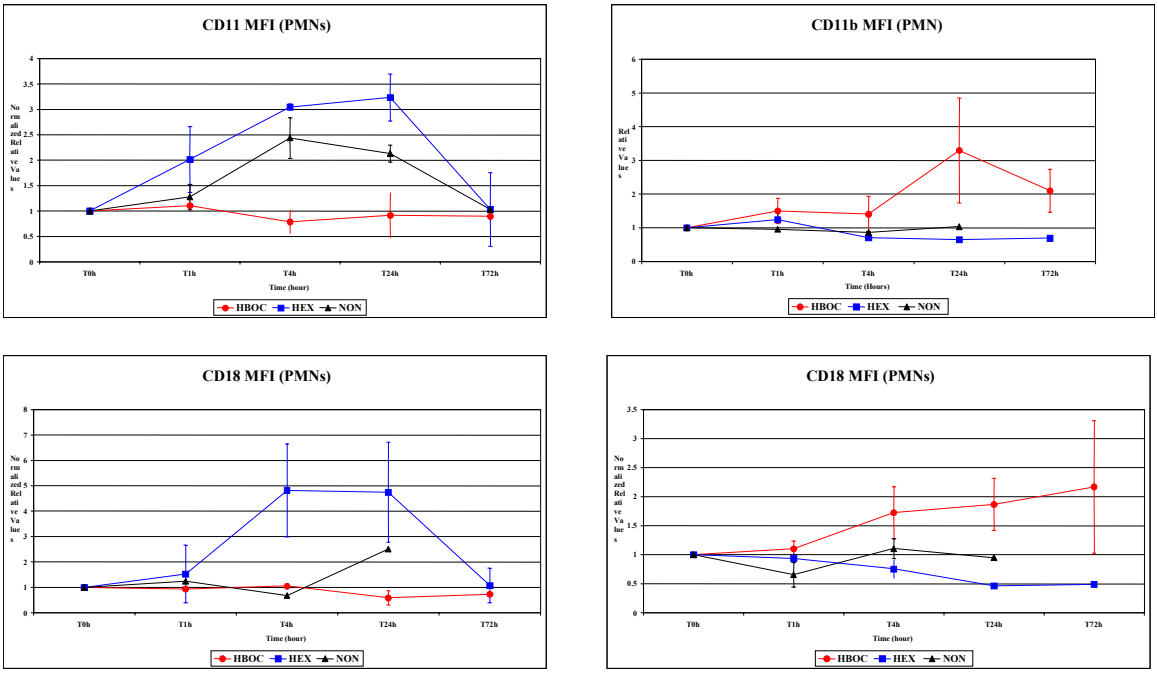


Figure 4. CD11 and CD 18 data from controlled hemorrhage model

In contrast to observations in the controlled hemorrhage model, in the swine HS model with uncontrolled hemorrhage, expression of PMN CD11b and CD18 and of lymphocytic CD49 increased ~2-3-fold in HBOC-201-pigs; PMN CD49 remained stable. As there were few survivors in the control groups in this severe HS model, treatment-effect comparisons across groups could not be completed. However, significant effects on adhesion marker expression were not obvious in NON- and HEX- resuscitated pigs (except possibly for PMN CD49).

5.0 DISCUSSION AND CONCLUSIONS

As 90% of war fatalities occur on the battlefield and 60% of potentially salvageable deaths are related to rapid exsanguination, immediate restoration of blood volume and O_2 content may be lifesaving, but expeditious blood transfusion is rarely available for combat casualties (especially in the asymmetric battlefield) [6]. Pre-evacuation standard of care relies on attempts at extrinsic hemostasis and resuscitation with asanguinous fluids, but complications of free radical generation, immune activation, reperfusion injury, irreversible shock, MOF, and coagulopathy and thrombopathy are common. Thus, a whole blood-like bridging volume replacement fluid with O_2 transporting properties as well as hemostatic, immunomodulating, antiapoptotic, and antioxidant properties that does not require a cold chain, is likely to improve outcome of HS casualties. Moreover, in keeping with requirements of operational agility, efficiency, and limited medical footprint in SeaPower 21 (i.e., Sea Basing), optimal stabilization of combat casualties is vital. Recent work in this area has led to the development of a comprehensive database of the physiological, hematological, immunological, and histopathological consequences of HS with controlled and uncontrolled hemorrhage in swine.

In these experiments, we have demonstrated that HBOC-201 improved hemodynamics and tissue oxygenation, decreased lactic acidosis, and improved survival in HS swine models. Despite moderate vasoactivity, hemorrhage was not increased in HS with uncontrolled hemorrhage due to solid organ injury.

TEG data from the controlled hemorrhage model suggest that in comparison with controls, HBOC-201-resuscitation may induce mild coagulopathy but decrease thrombopathy. The pattern of TEG data from the uncontrolled hemorrhage model was similar except that in comparison with HEX-resuscitation, TEG-CI was higher with HBOC-201, suggesting diminished coagulopathy. Taking together, these data suggest that the hemostatic effects of HBOC-201-resuscitation in HS are mixed and are unlikely to be clinically significant.

Furthermore, in moderately severe HS with controlled hemorrhage, resuscitation with HBOC-201 was "immuno-protective", preventing significant increases in leukocyte adhesion marker expression. In the severe HS model with uncontrolled hemorrhage, leukocyte adhesion markers increased despite HBOC-201 resuscitation, however, the relative increase, in comparison with controls, cannot be assessed in this model. As Ortegon showed in vitro that HBOC-201 stimulation of beta-2 integrin expression is dose-dependent, higher blood concentrations of HBOC-201 in the uncontrolled hemorrhage model may explain elevated adhesion marker expression. However, it is hypothesized that HBOC-201 may in fact have been immuno-protective but that early mortality in the comparator groups preclude such comparisons.

In comparison with HEX, HBOC-201 restored tissue oxygenation, decreased lactic acidosis, diminished pro-inflammatory neutrophil activation, and improved survival, without increased hemorrhage or deleterious effects on hemostasis, in controlled and uncontrolled HS models. Mild enhancement of HS-induced coagulopathic changes were observed. HBOC-201 increased PMN and lymphocyte adhesion marker expression and apoptosis, and elaboration of both pro- and anti-inflammatory cytokines. Clinical trials should be completed to definitively compare the efficacy and safety of HBOC-201 and traditional asanguinous resuscitative fluids for the resuscitation of HS casualties.

6.0 ACKNOWLEDGEMENTS

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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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