
# Evaluation of Ox66 Ingestion for Improvement of Oxygenation During Hemorrhagic Shock in Swine

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**EVALUATION OF OX66 INGESTION FOR IMPROVEMENT OF OXYGENATION DURING HEMORRHAGIC SHOCK IN SWINE**

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**14. ABSTRACT-** Hemorrhagic shock due to trauma is a major cause of morbidity and mortality on the battlefield. Providing optimal fluid resuscitation options will improve outcomes following severe trauma. A resuscitation fluid, or combination of fluids, that retains the desirable properties of whole blood are needed: These properties include fluid volume replacement, coagulation factors, and oxygen delivery. Ox66 is a novel, aluminum hydroxide compound that forms a clathrate that contains molecular oxygen. This oxygen can be delivered to oxygen deprived tissue sites during hemorrhagic shock. However, Ox66 is not refilled after releasing oxygen, and it does not carry waste products such as carbon dioxide. The purpose of this study was to test the efficacy of Ox66 in a hemorrhagic shock swine model with regards to survival, oxygen status measurements, and hemodynamics.

Male, swine had up to 40% of blood was removed over a 30-minute period to simulate hemorrhagic shock. One gram per kilogram Ox66 was delivered directly to the animal's stomach through oral gavage. A second dose of Ox66 was given one hour later. Control animals were treated similarly except they were given water through gavage following hemorrhage.

There were no significant differences between groups at baseline and only pH was significantly different following hemorrhage. Survival between groups was not significantly different by Fisher's exact test (p=0.999) or by log rank analysis (p=0.819) with 60% of control and 67% of Ox66-treated animals surviving to the end of observation. Secondary outcome measures (mixed venous oxygen saturation, blood pressure, peripheral blood oxygen saturation, partial pressure of oxygen in the blood, and tissue oxygenation) were not significantly different between groups as analyzed by ANOVA.

In conclusion, this pilot study demonstrated that Ox66 could safely and feasibly be utilized for resuscitation from hemorrhagic shock; however, no observable improvement in outcomes were attained with its' use. Further investigation is needed to determine its' efficacy.

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## 1.0 EXECUTIVE SUMMARY

Hemorrhagic shock due to trauma is a major cause of morbidity and mortality on the battlefield. Providing optimal fluid resuscitation options will improve outcomes following severe trauma. While fresh whole blood is the optimal resuscitation fluid to treat hemorrhagic shock, difficulties exist with its use including storage conditions, weight, possible contamination, and blood-type issues. A resuscitation fluid, or combination of fluids, that retains the desirable properties of whole blood are needed: These properties include fluid volume replacement, coagulation factors, and oxygen delivery. Ox66 is a novel aluminum hydroxide compound that forms a clathrate. The clathrate is comprised of approximately 66% oxygen bound within the aluminum cage. This oxygen can be delivered and unloaded to oxygen deprived tissue sites during hemorrhagic shock. Ox66 is available as a dried powder making it a favorable candidate for field and prolonged field care use. However, Ox66 is not refilled after releasing oxygen, and it does not carry waste products such as carbon dioxide. The purpose of this study was to test the efficacy of Ox66 in a hemorrhagic shock swine model with regards to survival, oxygen status measurements, and hemodynamics.

Male, Yorkshire-Landrace cross swine (70-90kgs) were anesthetized, instrumented, and had their spleens removed. After stabilization, 40% of blood was removed over a 30-minute period to simulate hemorrhagic shock. After intravenous administration of 500mL of Hextend, one gram per kilogram Ox66 was delivered directly to the animal's stomach through oral gavage. A second dose of Ox66 was given one hour later. Control animals were treated similarly except they were given water through gavage following hemorrhage. Hemodynamics were continuously recorded, while blood gases, blood chemistries, ROTEM, and CBCs were regularly measured throughout the three-hour observation period.

There were no significant differences between groups at baseline and only pH was significantly different following hemorrhage. Survival between groups was not significantly different by Fisher's exact test ($p=0.999$) or by log rank analysis ($p=0.819$) with 60% of control and 67% of Ox66-treated animals surviving to the end of observation. All secondary outcome measures (mixed venous oxygen saturation, blood pressure, peripheral blood oxygen saturation, partial pressure of oxygen in the blood, and tissue oxygenation) were not significantly different between groups as analyzed by ANOVA. An a priori power analysis was performed using an estimated 70% increase in survival to achieve 80% power and 0.05 significance. The difference observed here was only 7% indicating a lack of effect in this highly controlled laboratory experimentation.

In conclusion, this pilot study demonstrated that Ox66 could safely and feasibly be utilized for resuscitation from hemorrhagic shock; however, no observable improvement in outcomes were attained with its' use. Further investigation is needed to determine its' efficacy.

## 2.0 INTRODUCTION

Hemorrhage is associated with the majority of potentially survivable trauma deaths on the battlefield.[1,2] The goal of resuscitation from hemorrhagic shock is to restore end-organ perfusion and tissue oxygenation while simultaneously achieving definitive control of bleeding.[3] Whole blood is the ideal resuscitation fluid for hemorrhagic shock since it contains all of the necessary components for selective thrombosis at the site of injury to facilitate hemostasis and prevent the coagulopathy of trauma.[4] Blood also acts as an intravascular volume expander, restoring hemodynamics and improving cardiac performance and oxygen delivery. Finally, blood erythrocytes are efficient oxygen carriers, crucial for oxygen delivery to end organs that prevents the metabolic consequences of hemorrhagic shock.

Unfortunately, the use of whole blood on the battlefield is limited by a number of significant issues. The logistics of blood supply can be prohibitive as it requires refrigeration, is bulky to carry, heavy, and has a limited shelf life. Additionally, though rare, the use of blood for resuscitation carries the risk of blood-type mismatch and infectious complications.[5] A lightweight, shelf-stable, and easy to deliver blood substitute that is safe, effective, and logistically feasible could obviate many of these issues and replace or supplement the use of whole blood for the resuscitation of hemorrhagic shock in both civilian and military environments.

Many resuscitative fluids designed to fulfill some, or all of the functions of whole blood have been developed and implemented for management of hemorrhagic shock. Crystalloid and colloid solutions are the most common. Crystalloid solutions are composed of ions that are freely permeable across capillary membranes and include 0.9% (normal) saline and Lactated Ringer's. During the Vietnam War, research supported the use of large volumes of isotonic crystalloid solution to replace intravascular fluid loss and interstitial volume.[6,7] However, animal studies from the late 20$^{th}$ century demonstrated greater incidence of hyperchloremic metabolic acidosis and increased mortality associated with large volumes of isotonic saline infusion, leading to the widespread adoption of Lactated Ringer's solution as the resuscitation fluid of choice.[8,9] In the decades since, additional research has demonstrated the harmful effects of large-volume crystalloid resuscitation, including pericardial effusion, interstitial edema, derangement of cellular and immune function, and even the emergence of Acute Respiratory Distress Syndrome (ARDS). [10–13] This new information shifted crystalloid resuscitation use to research and increased interest in colloid resuscitation fluids.

Colloid fluids are composed of suspensions of molecules in a carrier solution that are incapable of crossing the capillary membrane due to the relatively larger size of the suspended molecules. Common colloid solutions include 4% albumin and hydroxyethyl starches. Research on colloids produced mixed findings. Animal studies demonstrated more rapid restoration of tissue perfusion, improved oxygen delivery, and reduced lung injury in colloid treated groups relative to crystalloids.[14–16] However, some studies demonstrated these differences did not contribute to a meaningful reduction in mortality for trauma patients and even suggested colloids were associated with an increase in mortality relative to crystalloid fluids, most commonly because of acute kidney injury or coagulopathy.[17–20] These results appear to be dependent on the specific properties of the colloid being used but are largely due to negative affect of colloid osmotic pressure on glomerular filtration rate, as well as affects to the coagulation cascade due to hemodilution.[20,21] Hypertonic fluids, such as 7.5% saline, were also investigated as a means of rapidly expanding plasma volume relative to other crystalloid and colloid solutions, and animal research suggested hypertonic saline with 6% dextran demonstrated promise as a resuscitation fluid.[22] However, a 2008 prehospital trial demonstrated that hypertonic saline with dextran increased early mortality and performed no better than normal saline in overall mortality.[23]

The elusiveness of an ideal resuscitation fluid has warranted investigation of various resuscitation strategies to determine whether the means of resuscitation can confer a survival advantage, such as hypotensive or delayed resuscitation. Both strategies have been shown to perform as well or better than standard fluid therapy strategies, with hypotensive resuscitation demonstrating a slight benefit, especially in cases where transport to definitive care may be delayed.[3,24–28] Beyond conventional resuscitative fluids and alternative resuscitative strategies, extensive research has also been conducted investigating the efficacy of blood component therapies and blood substitutes that replicate the function of blood, namely its oxygen-carrying capacity. Blood component therapy utilizes the various components of blood; specifically, packed red blood cells, fresh frozen plasma, and platelets; in various ratios for treatment of hemorrhagic shock. Blood component therapy still necessitates cross-matching between donor and recipient and presents many of the same issues with regards to storage as whole blood does but has the advantage of conserving resources by allocating components in ratios as needed. Various reviews of clinical studies have demonstrated that although there remains disagreement with regards to the ideal ratio of components for transfusion, commitment to a selected ratio followed by careful adherence to a standardized protocol for care may reduce mortality.[29–31]

Blood substitutes have been designed with the goal of fulfilling the functions of blood without the infection risk associated with blood transfusion. Polymerized hemoglobin solutions, also known as hemoglobin-based oxygen carriers (HBOCs), are universally compatible and shelf-stable blood substitutes. Multiple configurations, such as Hemopure, Polyheme, and HemAssist, have been developed and tested, but only Hemopure remains considered for clinical use.[3,32] Multiple studies have demonstrated an increased risk of myocardial infarction and mortality, as well as preclinical side effects including transient hypertension, gastrointestinal complications, and coagulopathy, associated with the use of HBOCs.[32,33] The mechanisms of toxicity with HBOCs are not well understood but are hypothesized to result from hemoglobin extravasation across blood vessel walls, nitric oxide scavenging, endothelial dysfunction, and oversupply of oxygen impeding hemostasis.[33,34] However, a recent study in 2015 advocated for reassessing the clinical use of all HBOCs based on a better understanding of the properties of the various classes of HBOCs, but they remain controversial.[35] In laboratory settings, Hemopure (HBOC-201) has been shown to perform as well as 6% hetastarch, a colloid fluid considered the military standard of care; and was demonstrated to be well-tolerated in human clinical trials.[36–39]

Despite these numerous advances in resuscitation from hemorrhagic shock, an ideal replacement for whole blood has yet to be discovered. However, it is possible that a combination of various components that fulfill the individual functions of blood will prove to be safe, efficacious, easily stored, and readily available. Ox66 is a novel polyoxygenated aluminum hydroxide compound. It is comprised of approximately 66% oxygen bound within a lattice-like structure which can be delivered to and unloaded at hypoxic tissue sites, potentially fulfilling the oxygen-carrying capacity of whole blood. However, unlike hemoglobin, Ox66 cannot bind oxygen again once used and therefore its application may be limited. Nonetheless, it is a soluble, lightweight powder that is benign for handling and use and requires no refrigeration or other special storage methods, making it feasible for rapid deployment. When used with an intravascular volume expander and a hemostatic compound, Ox66 could prove beneficial in a multifunctional resuscitation fluid and serve as a potential replacement for whole blood. In early cell culture and rodent studies, Ox66 was found to be both safe and effective as an oxygen carrier.[40,41] However, the application of Ox66 in a large-animal model that simulates battlefield hemorrhagic shock has not been examined.

This study had two goals: 1) to examine the efficacy and ability of Ox66 to deliver oxygen to end organs and mitigate metabolic shock following hemorrhage and 2) asses the basic safety of Ox66 for resuscitation of hemorrhagic shock. Ox66 has the potential to be more readily available and rapidly deployed for

resuscitation from hemorrhagic shock compared to other resuscitation fluids and substitutes available today. The primary and secondary outcomes of this study included survival, total fluid resuscitation requirement, oxygen debt, and other metabolic indicators of shock.

## 3.0 METHODS, ASSUMPTIONS, AND PROCEDURES

The experimental protocol was approved by the 59th Medical Wing Institutional Animal Care and Use Committee (IACUC). Experiments were performed at the 59th Medical Wing, United States Air Force, Office of the Chief Scientist, in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care and was conducted in accordance with guidelines established by the Public Health Service Policy on Humane Care and Use of Laboratory Animals and Office of Laboratory Animal Welfare. The procedures were performed by qualified personnel under the supervision of on-site veterinarians.

### 3.1 Animal Model

The model employed in this study was a controlled hemorrhage model previously used by our group and based on the Frankel model of variable, controlled hemorrhage.[42] Up to 40% of the estimated blood volume was withdrawn over thirty minutes to induce class IV hemorrhagic shock. Half of this volume was withdrawn over the first ten minutes of hemorrhage, and the other half was withdrawn over the remaining twenty minutes. This model of controlled hemorrhage more closely resembles a clinical presentation of hemorrhage occurring at a variable rate, thus yielding a more severe physiologic insult. This model was employed according to the experimental design shown in Fig. 1

### 3.2 Animals

Male Yorkshire Landrace cross swine (Sus scrofa, 70-90kg) were obtained from a single local, USDA-licensed vendor. Animals were housed and fed in 59th Medical Wing facilities in accordance with operating instructions governing animal housing (40V-013-Feeding and Watering Schedules and 40V-014-Quarantine and Stabilization of Animals). Animals were allowed to acclimate to the facility for at least 7 days prior to surgery with free access to food and water. Feed was withheld 12 hours prior to surgery to reduce the likelihood of aspiration during intubation and anesthesia.

### 3.3 Anesthesia and Instrumentation

Animals were sedated via an intramuscular injection of Telazol (4.4mg/kg) and subcutaneous ketamine (2.2mg/kg). An intramuscular injection of Buprenex (0.01mg/kg) was also given as pre-emptive analgesia. Animals were intubated endotracheally using a laryngoscope and cuffed endotracheal tube held in position by roll gauze. Placement was confirmed by auscultation over both lung fields and mid-epigastrium, as well as assessment of $ETCO_2$ waveform. Anesthesia was induced via mask with 2-4% isofluorane, weaned down to 1-2.5% during the protocol to maintain a Mean Alveolar Concentration (MAC) of 1.2-2.0. Fraction of inspired oxygen ($FiO_2$) was set between 40 and 60%, and slowly weaned to 21% (atmospheric air) by the start of the 10-minute stabilization period. The End Tidal $CO_2$ was kept between 35 and 45mmHg until the 10-minute stabilization, maintained with an initial tidal volume of 7-10mL/kg and adjusted as needed. Once hemorrhage was initiated, ventilator settings were not adjusted in order to better asses the ability of the Ox-66 to improve oxygenation in the animal. Animal temperature was maintained between 37°C and 39°C using heating adjuncts as needed.

When anesthetized, electrocardiography, physiological monitoring, blood sampling, and vascular access instrumentation were prepared. Percutaneous blood sampling and vascular access lines were placed using ultrasound guidance or by surgical cutdown if needed. The following sites were cannulated with an 8.5Fr catheter: 1) right carotid artery for intra-aortic blood pressure (BP) monitoring via a micromanometer-tipped catheter (Millar Inc. Houston, TX USA) as well as blood sampling; 2) right external jugular vein for pulmonary artery BP, cardiac output, mixed venous oxygen saturation (SvO2), and cardiac temperature

monitoring via a Swan-Ganz thermodilution catheter (Edwards Lifesciences, Irvine, CA USA); 3) left external jugular vein for fluid infusion, and 4) right femoral artery for exsanguination. Additionally, an orogastric (OG) tube was placed into the stomach via the oropharynx for oral administration of the assigned treatment. Rectal temperature was monitored via a rectal temperature probe inserted during initial animal preparation. A disposable peripheral capillary oxygen saturation ($SpO_2$) monitor was placed on the front right hoof. Near Infrared Spectroscopy (NIRS) pads were placed on the left and right medial thighs, on top of the right kidney, as well as the pectoralis muscle to monitor muscular tissue oxygenation.

### 3.4 Surgery

Following line placement and instrumentation, a splenectomy was performed via a midline laparotomy. The *Sus scrofa* spleen is contractile and theoretically provides autotranfusion following injury that may limit the translatability of the results to clinical practice.[43] After the spleen was removed, the OG tube was advanced just distal to the esophageal sphincter to insure treatment administration into the stomach, and placement was confirmed via physical examination by the surgeons. A cystostomy was also performed for placement of a Foley catheter to prevent the physiological effects of a distended urinary bladder. The peritoneum and anterior abdominal wall were closed while allowing a small section of the distal liver lobe to remain exposed outside of the abdomen for placement of a Phosphorescence Quenching (PQ) probe. A small section of the front and hind limb muscles were also dissected and exposed for placement of two additional PQ probes. Phosphorescence Quenching utilizes non-contacting, oxygen-sensing probes to directly measure tissue oxygenation. Once all equipment was placed and the abdomen was closed, a ten-minute stabilization period was observed, and baseline measurements were collected.

### 3.5 Controlled Hemorrhage

After the ten-minute stabilization period, a controlled hemorrhage via the right femoral artery was initiated. Half of the estimated blood volume to be hemorrhaged (40% total) was withdrawn over ten minutes using a Masterflex peristaltic pump and tubing (Cole-Parmer, Vernon Hills, IL USA). The remaining volume was withdrawn over an additional 20 minutes, for a total hemorrhage period of 30 minutes. If at any point during the hemorrhage the Mean Arterial Pressure (MAP) dropped below 30mmHg, the hemorrhage was paused until the MAP rose above 33mmHg, at which point the hemorrhage was resumed.

### 3.6 Intervention

Following the hemorrhage, the animal was left in a period of shock for up to 15 minutes, during which no treatment was initiated. If during the 15 minutes the MAP dropped below 20mmHg, treatment was initiated (T0). The animal was randomized to either the experimental (Ox-66) or control (water – bottled drinking water) group using the sealed envelope method. Animals in both groups were initially given a 500mL intravenous bolus of Hextend (Pfizer, New York, NY) via the left external jugular vein using a Masterflex peristaltic pump at 100mL/min. After giving Hextend, a bolus of either Ox-66 (0.2g/mL in water) or water was administered by oral gavage. This bolus was delivered rapidly (~ 3 min) through a 500 mL syringe and feeding tube at a volume of 5mL/kg. After the first oral bolus, the animal was allowed to stabilize for 5 minutes, after which Hextend infusion at 100mL/min was resumed if the systolic blood pressure dropped below 90mmHg. Up to an additional 500mL was infused throughout the three-hour observation if needed. The second treatment bolus was administered at T65, one hour after the first bolus. Following treatment, the animal was observed for up to three hours after the initial intervention (T0), after which the animal was euthanized. If at any point during observation the animal met death criteria (MAP<20mmHg and EtCO2<15mmHg), the experiment was discontinued, and the animal was euthanized.

### 3.7 Post Experimental Procedures
At the end of the protocol, subjects were euthanized by a trained laboratory technician with an intravenous (IV) injection of Pentobarbital (100mg/kg) in accordance with the American Veterinary Medical Association euthanasia guidelines. Cessation of vital signs was confirmed via auscultation of the lungs and heart. The abdomen was reopened for gross examination of any damage to the stomach or digestive track due to the oral administration of Ox-66.

### 3.8 Data Acquisition
A PowerLab data acquisition system (ADInstruments, Colorado Springs, CO USA) was used to record data points for intra-aortic arch BP using a Millar micromanometer-tipped pressure catheter. This data was also used to calculate systolic, diastolic, and mean arterial pressures. These values were recorded as mean values every five seconds. This system also recorded live pulmonary arterial blood pressure, continuous cardiac output, cardiac temperature, and mixed venous oxygen saturation from the Swan-Ganz catheter. Other variables recorded included heart rate, end tidal carbon dioxide (EtCO2), peripheral capillary oxygen saturation (SpO2), NIRS data, and rectal temperature.

Interstitial oxygen tension ($P_{ISF}O_2$) was collected from three sites using a fiber-optic data acquisition system for phosphorescence quenching (PQ; Song Biotechnologies, Baltimore, MD USA). Two superficial skeletal muscles (forelimb extensor and hindlimb adductor femoris) and a superficial portion of the distal liver capsule were saturated with a phosphorescence probe (Oxyphore R0; Frontier Scientific, Newark, DE USA) and isolated from the atmosphere with an oxygen impermeable barrier film (CB-100; Krehalon Limited, Japan). Fiberoptic leads were positioned above each prep and shrouded to prevent light contamination. Excitation was at 524 nm and emission collected at >650 nm. Data in the form of phosphorescence decay rates were fitted to oxygen-calibrated standard curves and reported as mmHg. Sampling occurred at baseline (BL), every five minutes during hemorrhage (H), at the end of bleed (EoB), after ten minutes of shock (S10), every five minutes of resuscitation (T0 – T15) and every ten minutes during the post-resuscitation observation phase.

Arterial blood samples (~1mL) were collected from the right carotid artery at the following time points: prior to hemorrhage (baseline), following hemorrhage and the 15-minute stabilization (T0), and at T15, T30, T45, T60, T90, T120, T150, and T180. Additional arterial blood samples (~10mL) were drawn at baseline, T0, T60, T120, and T180 for blood chemistries (Aspartate Aminotransferase, Blood Urea Nitrogen, Ca, Cl, Creatinine, Creatine Kinase, Potassium, Uric Acid).

### 3.9 Outcomes
The primary outcome of this study was survival throughout the entirety of the experiment. Secondary outcomes included physiological and metabolic parameters (arterial blood gas, HR, BP, and blood chemistries), measures of oxygenation (SpO2, SVO2, NIRS, PQ), and resuscitation requirements.

### 3.10 Data Analysis
Data is presented as mean ± standard deviation for continuous variable or by percent for categorical variables. Significance was assessed by Student's t-test or by repeated measures analysis of variance for time series. For PQ data, two-way ANOVA with Dunnet's and Fischers LSD posthoc tests were used to for intragroup comparisons to baseline and intergroup, respectively. All statistics were performed using Microsoft Excel 2013, SigmaPlot 13, or Graphpad Prism.

## 4.0 MAJOR EVENTS/MILESTONES/SUCCESS
In preparation for the execution of this project,

- IACUC Approval – 11 Jun 19
- All experimental procedures completed – 23 Sep 20
- Data Analysis – 15 Jan 21

## 5.0 RISK ASSESSMENT

### 5.1 Risk Analysis:

Significant risks:

- COVID19 and associated effects had a substantial effect on nearly every aspect of the project
- Delays due to alteration of Ox66 from an IV infusible form to a digestible form
- Delays due to procurement of Ox66
- Delays due to obtaining CRADA with Hemotek and with Song Biotechnologies

Impact of delays: animal procedures were initiated near end of period of performance. This delay caused an inability to change experimental protocol due to model development assessment. However, procedures were completed on time.

### 5.2 Technical Challenges:

The objective of this project was to test the Ox66 product in a model of controlled hemorrhagic shock. Changes in formulation of Ox66 to an ingestible form during the period of performance led to a significant challenge in creating a real-world scenario where oral gavage was performed during hemorrhagic shock.

## 6.0 TRANSITION PLAN

### 6.1 Military Relevance

This proposal focuses on treatment strategies for wound management to extend the golden hour and resuscitate from hemorrhagic shock. This is directly relevant for combat casualty care in the far forward military environment. It is aimed at the 2017 AFMS gap #1: Far Forward Blood, Blood Components, Blood Substitutes.

### 6.2 Transition Strategy

If successful, the compound studied here, Ox66, will undergo further testing in 6.3/6.4 research with realistic models of injury using current clinical practice guidelines for resuscitation (TCCC and JTS guidelines).

## 7.0 RESULTS

### 7.1 Baseline Values

Fifteen animals were enrolled in the study. Three were used as protocol development subjects in order to optimize the model and delivery of Ox66 in this model. One animal was excluded due to an inability to survive the hemorrhage before beginning of treatment. The remaining eleven subjects were split between the water (n=5) and the Ox66 (n=6) groups. Baselines were similar with no significant differences between groups (Table 1). All animals were male and weighed 81.2 ± 5.6 kg.

### 7.2 Hemorrhage and Post Hemorrhage Values

As stated above, all animals but one survived the hemorrhagic shock period. However, all but one animal in the Ox66 group had to have the hemorrhage paused due to the MAP falling below 30mmHg. These

pauses resulted in a less than 40% hemorrhage in the majority of animals. One animal in the water group and two animals in the Ox66 group reached MAP < 20mmHg, but none qualified for death criteria as described in section 3.6 Intervention.

Post hemorrhage values before intervention are shown in Table 2. Results were similar between groups with only pH values significantly different between groups. Total hemorrhage volume was 1675 ± 382mL corresponding to 30.7 ± 6% of the estimated blood volume. This amount of hemorrhage resulted in an overall MAP of 30.7 ± 10.1mmHg.

The controlled hemorrhage performed here resulted in clear indications of hemorrhagic shock in both groups. MAP, $EtCO_2$, potassium, lactate, and base excess were all significantly different than base line in both groups. Additionally, no significant differences were observed at the end of the hemorrhagic shock period between groups.

### 7.3 Survival

There were no significant differences between groups with regards to animal survival to the end of the observation period. Figure 2 shows the Kaplan-Meier curve over the course of the experiment. Log rank analysis revealed a nonsignificant p-value of 0.819 and Fisher's exact test of group survival was similarly not significant ($p = 0.999$).

### 7.4 Hemodynamic and Pulmonary parameters

No significant differences were observed between groups with respect to MAP, pulmonary artery (PA) MAP, $SpO_2$, $EtCO_2$, $SvO_2$ or any of the NIRS locations (Figure 3 and Figure 4). Blood pressures in both groups recovered quickly after the administration of Hextend reaching the predetermined resuscitation cutoff of 60mmHg by about 20 minutes after initiation of Hextend infusion. $EtCO_2$ also quickly rose to above baseline values following Hextend administration and returning to normal by the end of the three-hour observation period. Oxygen saturation remained largely unchanged during the entire experimental period, as well as mixed venous oxygen saturation as measured using the pulmonary artery catheter. No observable response to Ox66 was observed following either the $T_5$ or $T_{65}$ bolus.

Tissue oxygenation measured using NIRS at the right pectoralis muscle, right kidney, left thigh, and right thigh showed no differences between groups (Figure 5). All measurements using NIRS showed a decrease in oxygenation during hemorrhagic shock period followed by an increase shortly after Hextend infusion. No increase in oxygenation was seen following the two Ox66 boluses. There appears to be a trend in the kidney NIRS toward improved oxygenation in the Ox66 group, but statistical analysis could not be performed in these animals due to missing data in the water treated animals.

### 7.5 Blood Lab Values

Arterial blood gas analysis was performed throughout the experiment and is shown in Figure 5. No significant differences were observed in $pO_2$, potassium, lactate, and base deficit. While $pO_2$ remained nearly steady in both groups, potassium, lactate, and base deficit all respond to both the hemorrhage and to Hextend resuscitation. Similar to the oxygen saturation, no observable increase in oxygenation was seen following either Ox66 bolus.

Blood chemistry values are presented in Figure 6. There were no significant differences in any parameter tested. Creatinine (CRE) and Blood Urea Nitrogen (BUN) had steady increases in both groups with no

change due to Hextend or Ox66. Creatine Kinase (CK) decreased early on during resuscitation but eventually trended upwards towards the end of the observation period. Aspartate Aminotransferase (AST) remained steady in both groups until the last time point where Ox66 animals saw a large, but not significant increase in AST levels.

### 7.6 Interstitial $PO_2$ of Skeletal Muscle and Liver

Resuscitation with Hextend generally improved $P_{ISF}O_2$ in skeletal muscle and liver but impacts were non-significant and statistically underpowered. The Ox66 group ended hemorrhage with numerically higher liver $P_{ISF}O_2$ values (Fig. 7), which became briefly significant against Water at onset of Hextend infusion (T0). No treatment effect of Ox66 gavage at t5 was noted in liver, but T15 showed significantly higher $P_{ISF}O_2$ in the forelimb compared to Water (Fig. 8A). For the hindlimb skeletal muscle, Ox66 trended higher than Water after T15 ($p=0.10$ at T45), but never reached significance (Fig. 8C). The T65 bolus had no effect on tissue oxygenation, which generally decayed after T65 for both groups.

## 8.0 CONCLUSION / DISCUSSION

Hemorrhage is associated with the majority of the potentially survivable deaths on the battlefield[1] and the ability to adequately oxygenate these patients is critical for recovery. Whole blood is the best choice for resuscitation due to it replacing exactly what is lost during hemorrhage. However, whole blood and blood components present many logistical problems on the battlefield including difficulties in transport, contamination, blood-type compatibility, and shelf life. Alternatives to blood exist that are able to transport oxygen through the blood including HBOCs and perfluorocarbons (PFCs) but have not been supported enough to replace the standard of care. Another possibility for replacing the use of whole blood is to deliver oxygen directly via a chemical compound such as Ox66.

This project was undertaken as a pilot study to determine the ability of Ox66 to deliver oxygen during resuscitation from hemorrhagic shock when oxygen demand is greatest. We utilized a commonly used model of controlled hemorrhage to assess Ox66 benefits during fluid resuscitation. Fluid resuscitation is generally delivered directly into the blood through an intravenous (IV) or intraosseous (IO) route. Unfortunately, an IV infusible form of Ox66 was not available during the experimental phase of this study. Therefore, an ingestible form of Ox66 was used instead.

The main outcomes of this study were survival and markers of oxygenation. Despite the presence of significant markers of hemorrhagic shock, none of the factors examined here were statistically different between groups. We did not observe a noteworthy increase in oxygen in any measurement ($SpO_2$, $pO_2$, NIRS, etc.) that would correspond to an increase in circulating oxygen following the delivery of Ox66. Only a minor increase in interstitial oxygen was observed in the forelimb at T15 but not at other time points or tissues examined. Furthermore, a strong trend in elevation of the liver enzyme, AST, may indicate liver toxicity that needs to be further examined in swine or other model organisms.

Administering Ox66 thorough oral gavage presented some limitations that will need to be addressed moving forward for Ox66 to be utilized on the battlefield. Gavage provides an inherit danger of aspiration to an injured service member. It also may prove difficult to implement in battlefield scenarios based on injury type and location. User experience of the product shows that it is both difficult to re-suspend into suspension and difficult to push through the feeding tube. Finally, we observed a large amount of unabsorbed Ox66 in the stomach and small intestine. Although the cause for this lack of absorption in the animals observed in this study is not known, it may be related to the physiologic state of animals during hemorrhagic shock.

This study had several additional limitations. The injury produced here was due to a controlled hemorrhage, which does not reflect real world injuries. Another type of injury or polytrauma could have resulted in a larger oxygen debt and thereby provide a bigger opportunity to observe increases in oxygen from Ox66 delivery. As this was a pilot study, animal numbers were low and may have masked statistically relevant differences. However, increases in oxygen levels are easily observable in laboratory measurements especially $pO_2$ of blood. Additionally, the use of Hextend was likely necessary to support survival throughout the entirety of the observation, but its use may have masked any potential benefit the Ox66 provided compared to just water alone.

In aggregate, the results here do not provide any evidence that Ox66 delivered through oral gavage is able to deliver oxygen in a meaningful way during hemorrhagic shock. Markers of hemorrhagic shock were observed in both groups indicating the model produced a physiologically relevant injury. Ox66 in other forms may fulfill the goal of supplementing oxygen during hemorrhagic shock. Additionally, Ox66 may provide benefits in other indications including the militarily relevant condition of lung injury such as Acute Respiratory Distress Syndrome (ARDS).

## 9.0 DELIVERABLES

At this time, this final report is the only deliverable. This research may result in a peer-reviewed publication that could offer supportive data for large-scaled studies in the future as Ox66 represents a potential long-term deliverable of a field-capable resuscitation fluid or adjunct. However, results obtained from this research did not provide any evidence that Ox66 delivered through oral gavage is able to deliver oxygen in a meaningful way during hemorrhagic shock. Partners working on this technology continue to work through the additional formulations to improve the technology and will continue to inform of any new developments for future collaborative considerations.

## 10.0 COST

This work was selected and funded by the Air Force Medical Support Agency (AFMSA) funding under project code number AC19CR01. This project received AC9 funds on 19 December 2019 ($205k).

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## 12.0 FIGURES AND TABLES

**Table 1. Baseline values**

| | Water | Ox66 | *p*-value |
|---|---|---|---|
| n | 5 | 6 | |
| Weight (kg) | 81.4 ± 5.1 | 81.0 ± 6.5 | 0.914 |
| MAP (mmHg) | 67.8 ± 3.4 | 67.2 ± 6.1 | 0.843 |
| EtCO2 (mmHg) | 42.3 ± 2.1 | 43.0 ± 2.4 | 0.640 |
| pH | 7.49 ± 0.01 | 7.478 ±0.03 | 0.240 |
| pO2 (mmHg) | 94.5 ± 12.5 | 106 ± 16.9 | 0.227 |
| Potassium (mmol/L) | 4.3 ± 0.3 | 4.2 ± 0.2 | 0.517 |
| Lactate (mmol/L) | 2.2 ± 0.7 | 2.4 ± 0.3 | 0.529 |
| Base excess (mmol/L) | 7.2 ± 1.9 | 5.4 ± 1.3 | 0.097 |
| Hemoglobin g/dL | 9.6 ± 0.5 | 9.8 ± 0.8 | 0.567 |
| Forelimb $P_{ISF}O_2$ (mmHg) | 22.3 ± 5.8 | 27.0 ± 4.7 | 0.815 |
| Liver $P_{ISF}O_2$ (mmHg) | 44.1 ± 8.9 | 34.6 ± 9.1 | 0.476 |
| Hindlimb $P_{ISF}O_2$ (mmHg) | 10.2 ± 2.9 | 14.4 ± 4.9 | 0.484 |

**Table 2. Post Hemorrhage Values**

| | Water | Ox66 | *p*-value |
|---|---|---|---|
| N | 5 | 6 | |
| Hemorrhage (mL) | 1635 ± 449 | 1707 ± 357 | 0.772 |
| Hemorrhage (%) | 29.9 ± 7.3 | 31.4 ± 5.8 | 0.706 |
| MAP (mmHg) | 26.2 ± 3.2$^{\#\#}$ | 34.5 ± 12.6$^{\#\#}$ | 0.385 |
| EtCO2 (mmHg) | 31.8 ± 5.5$^{\#}$ | 32.2 ± 7.6$^{\#}$ | 0.924 |
| pH | 7.48 ± 0.04 | 7.40 ± 0.05$^{\#}$ | 0.021* |
| pO2 (mmHg) | 105.6 ± 23.6 | 92.1 ± 21.3 | 0.342 |
| Potassium (mmol/L) | 6.5 ± 0.09$^{\#\#}$ | 6.3 ± 1.6$^{\#}$ | 0.816 |
| Lactate (mmol/L) | 7.74 ± 3.0$^{\#\#}$ | 10.0 ± 1.6$^{\#\#}$ | 0.137 |

| | | | |
|---|---|---|---|
| Base excess (mmol/L) | -0.3 ± 4.8# | -4.4 ± 2.4## | 0.101 |
| Hemoglobin g/dL | 8.46 ± 0.76# | 8.53 ± 1.3 | 0.913 |
| Forelimb $P_{ISF}O_2$ (mmHg) | 8.0 ± 2.6 | 9.9 ± 5.3# | 0.409 |
| Liver $P_{ISF}O_2$ (mmHg) | 4.4 ± 2.0# | 15.9 ± 6.8 | 0.158 |
| Hindlimb $P_{ISF}O_2$ (mmHg) | 7.3 ± 4.0 | 4.9 ± 2.1 | 0.620 |

*, $p < 0.05$ Water vs Ox66; #, $p < 0.05$ vs baseline; ##, $p < 0.01$

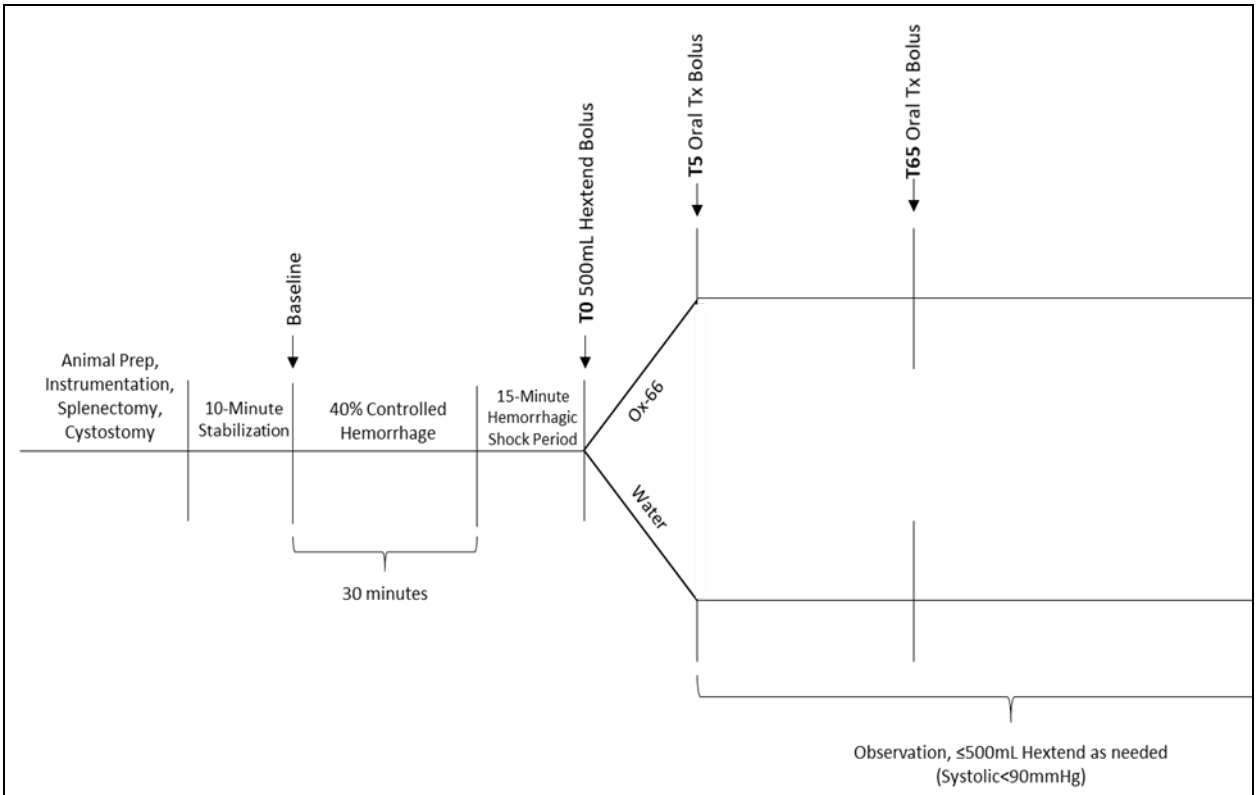


**Figure 1. Protocol Timeline.** After a 15-minute stabilization following the thirty-minute hemorrhage, the animal was randomized to either the experimental (Ox-66) or control (water) group. Animals in both groups immediately received a 500mL intravenous bolus of Hextend at 100mL/min, after which an oral bolus of either Ox-66 or water was given. The animal was then observed for up to 3 hours, during which the animal was given up to an additional 500mL of Hextend if the systolic blood pressure dropped below 90mmHg at any point. A second oral bolus was administered one hour after the first. The experiment was discontinued at the end of the three-hour observation, or if the animal met death criteria at any point throughout (Mean Arterial Pressure<20mmHg and EtCO2<15mmHg). See text for details

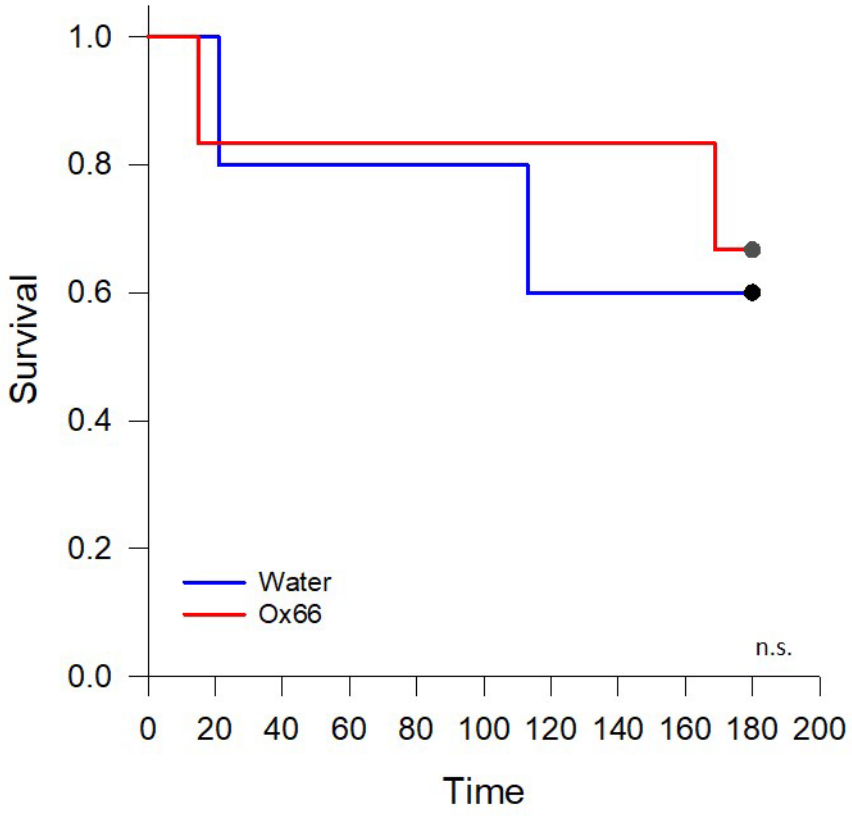


**Figure 2** Kaplan Meier Plot.

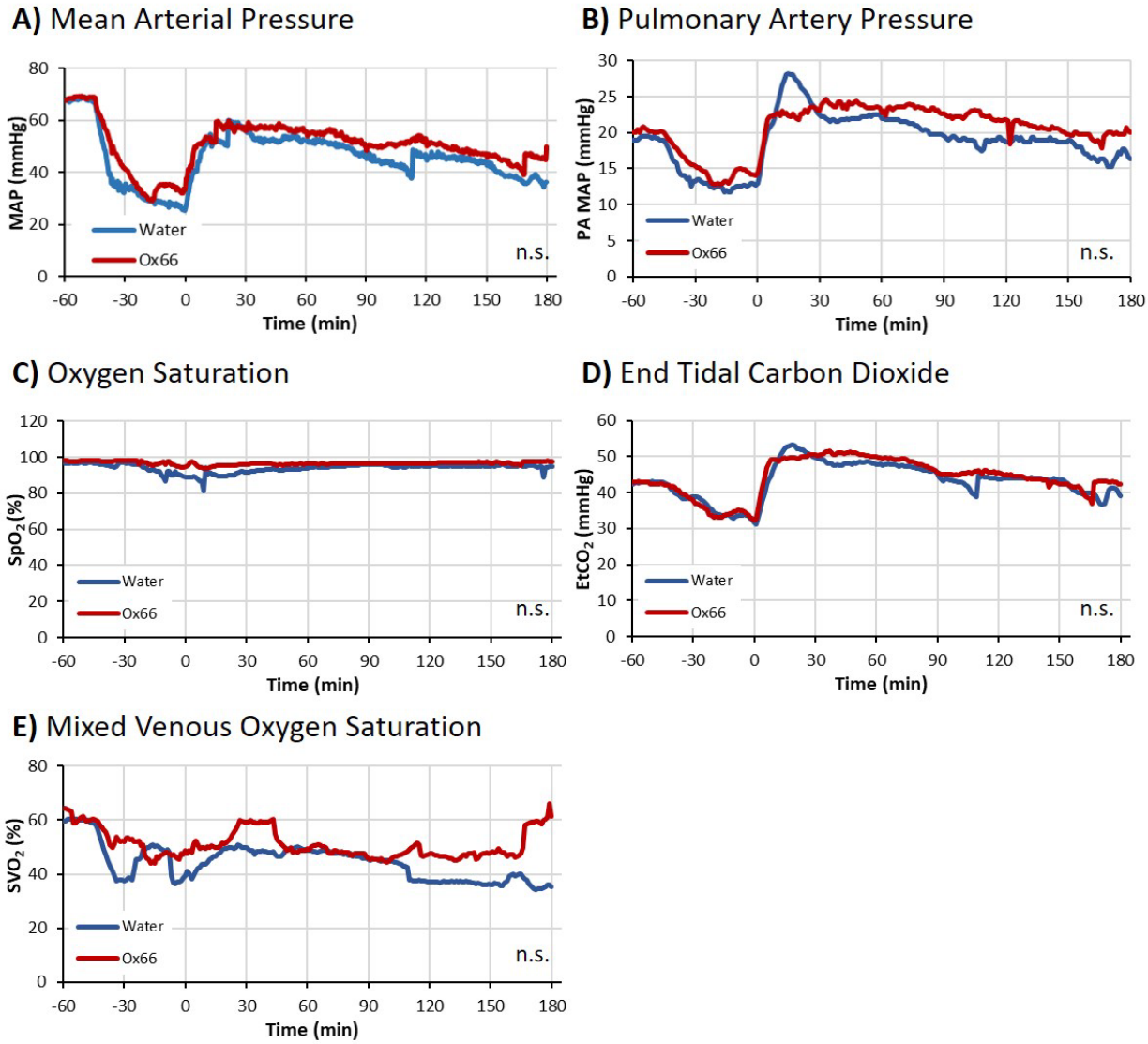


**Figure 3.** Hemodynamic Values. $T_{45}$ corresponds to the initiation of hemorrhage. $T_0$ is the start of resuscitation. Ox66 was administered via oral gavage at $T_5$ and $T_{65}$. Error bars removed for clarity. MAP, Mean Arterial Pressure; PA, Pulmonary Artery; SpO2, Oxygen saturation; EtCO2, End-tidal Carbon Dioxide; SvO2, Mixed Venous oxygen

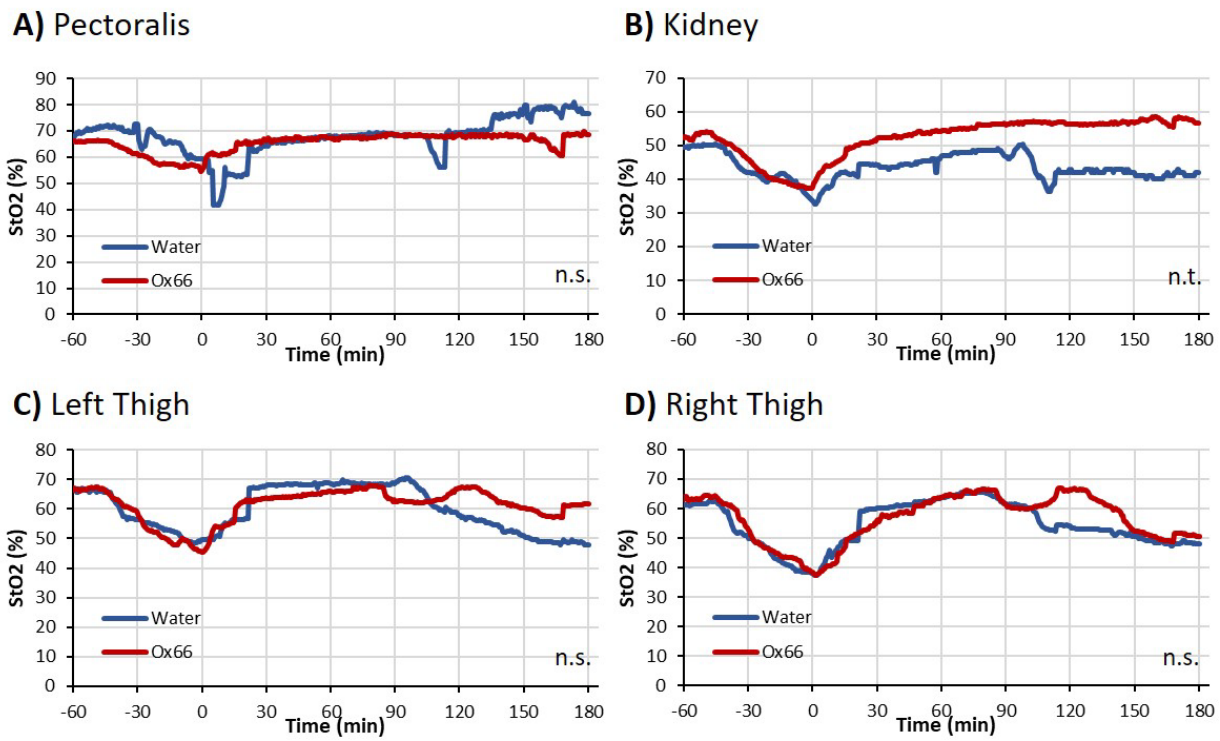


**Figure 4.** Tissue Oxygenation. $T_0$ is the start of resuscitation. Ox66 was administered via oral gavage at $T_5$ and $T_{65}$. Error bars removed for clarity. Kidney NIRS were not tested due to missing data. StO2, Tissue oxygen saturation; n.t. not tested

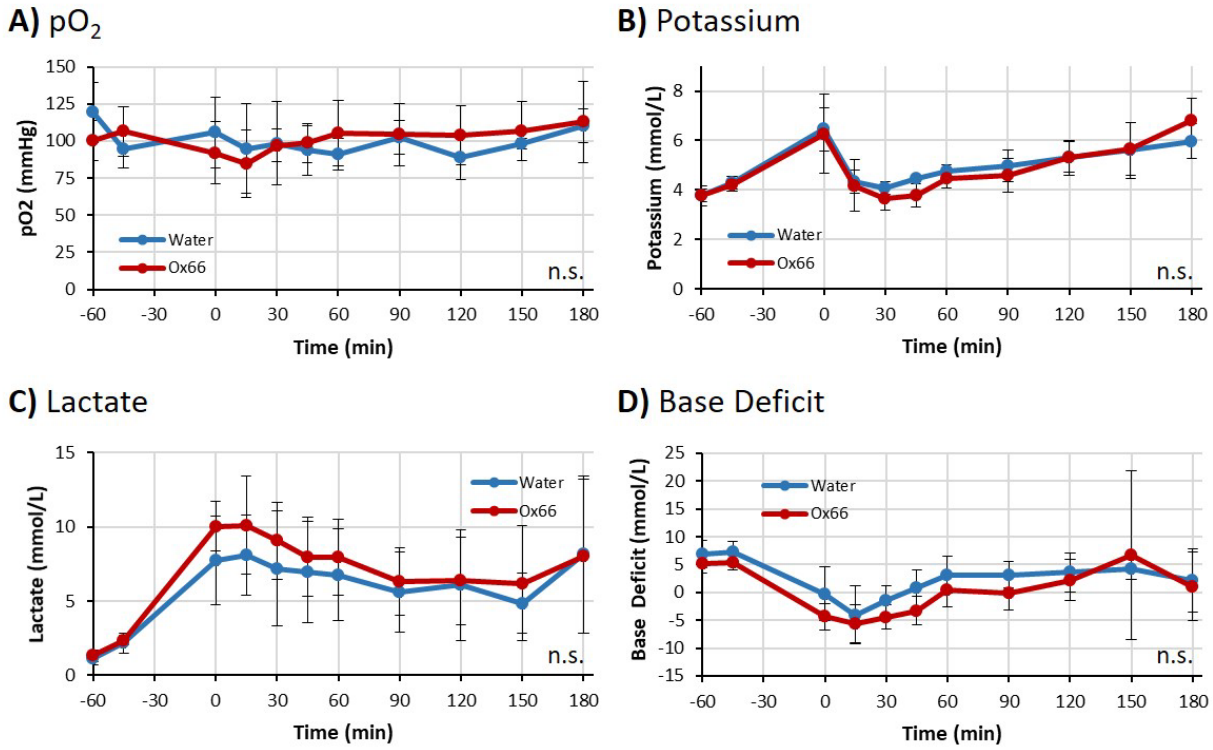


**Figure 5.** Blood Gas Values. $T_{45}$ corresponds to the initiation of hemorrhage. $T_0$ is the start of resuscitation. Ox66 was administered via oral gavage at $T_5$ and $T_{65}$.

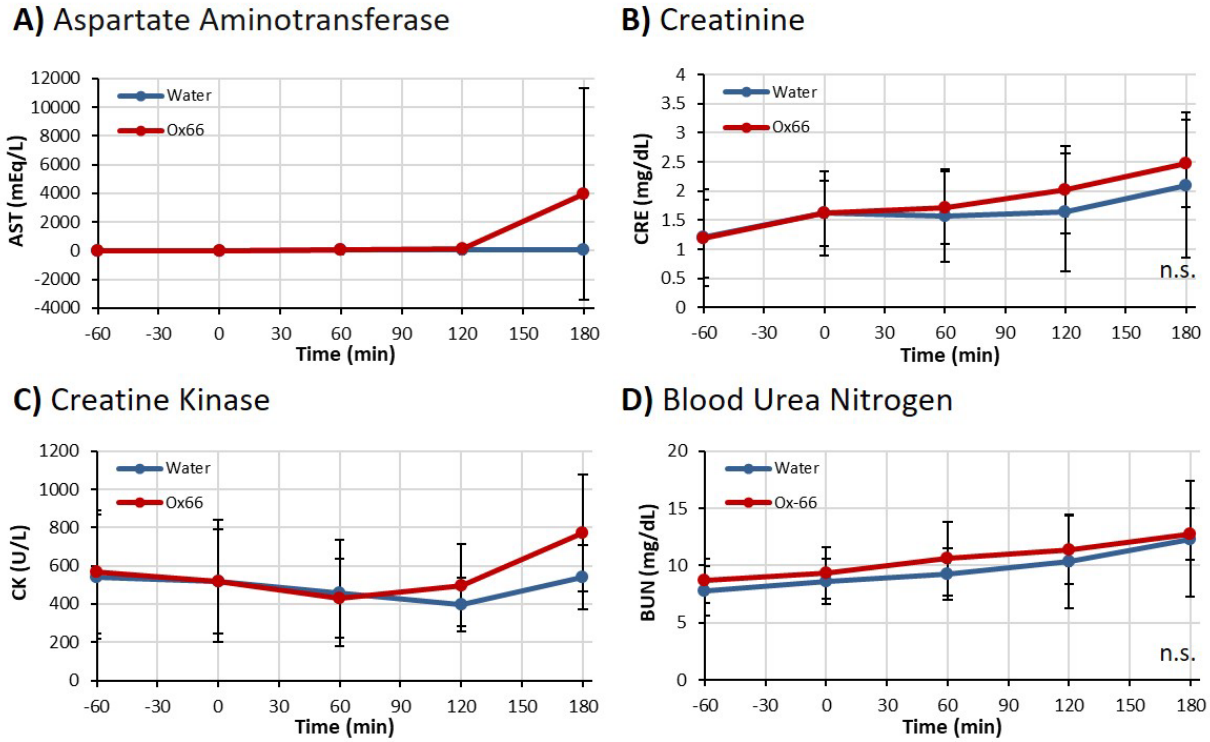


**Figure 6.** Blood Chemistry Values. $T_{45}$ corresponds to the initiation of hemorrhage. $T_0$ is the start of resuscitation. Ox66 was administered via oral gavage at $T_5$ and $T_{65}$. AST, Aspartate Aminotransferease; CRE, Creatinine; CK Creatine Kinase; BUN, Blood Urea Nitrogen

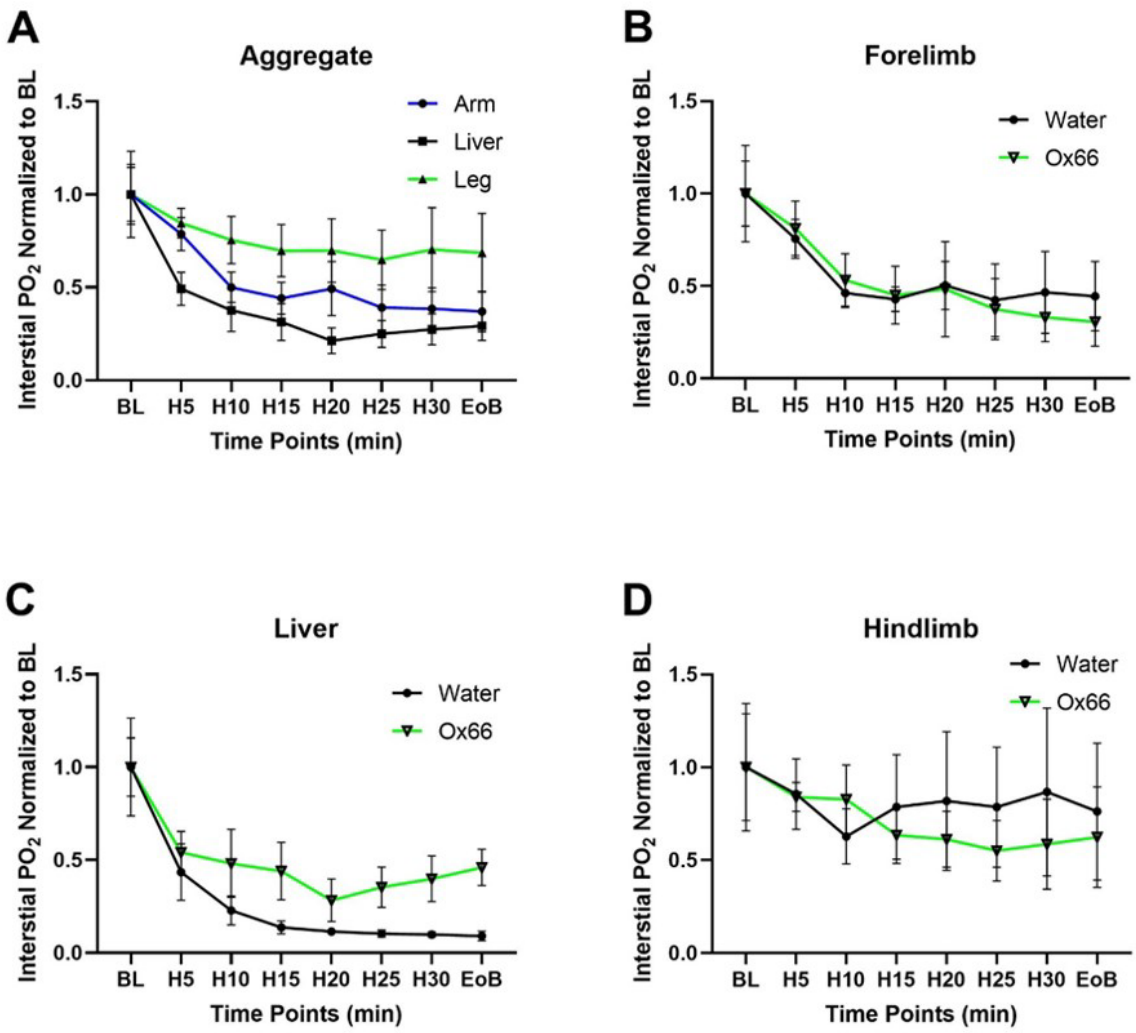


**Figure 7: Hemorrhage $P_{ISF}O_2$.** The first ten minutes involved a rapid, 20% blood volume withdrawal. Up to an additional 20% was withdrawn over the next 20 minutes. A: Since all animals were treated equally during hemorrhage, this shows a breakdown of tissue oxygenation between organs for both treatment groups. B: $P_{ISF}O_2$ profile for the forelimb extensor muscle. C: Superficial liver. D: Hindlimb adductor femoris. All three tissues were measured simultaneously for each animal. Raw data were normalized to baseline values. N=5 for water and 6 for Ox66. Data are mean ± SEM. BL- Baseline, Hn-hemorrhage time point in min, EoB-end of bleed.

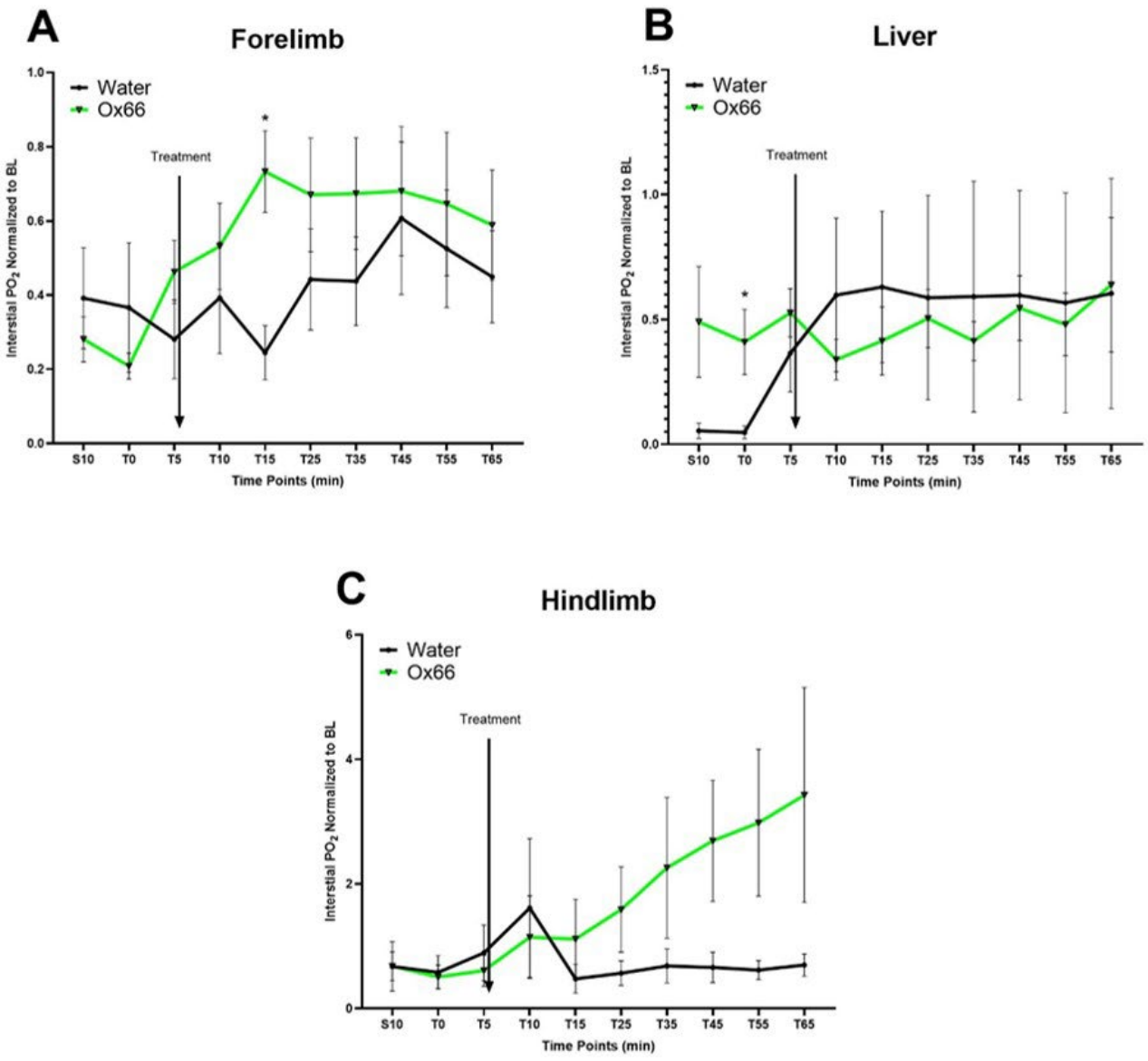


**Figure 8: Resuscitation $P_{ISF}O_2$.** S10 preceded the onset of Hextend resuscitation (T0) by 5 min. Fluid gavage treatment was administered immediately following T5 and fluid resuscitation ended (for most animals) by T15. Animals were given a second fluid bolus after T65 and tracked until demise or T180 (not shown), but it did not influence $P_{ISF}O_2$. A: $P_{ISF}O_2$ profile for foreleg extensor muscle. B: superficial liver. C: hindlimb adductor femoris. All three tissues were measured simultaneously for each animal. Data are mean ± SEM normalized to baseline (Fig. 7). N= 5 and 6 for water and Ox66, respectively. * $p<0.05$ v Water.

## 13.0 LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

| | |
|---|---|
| ARDS | Acute Respiratory Distress Syndrome |
| AST | Aspartate Aminotransferase |
| BUN | Blood Urea Nitrogen |
| CRE | Creatinine |
| CK | Creatine Kinase |
| $EtCO_2$ | End tidal Carbon Dioxide |
| HBOC | Hemoglobin-based oxygen carrier |
| IO | Intraosseous |
| IV | Intravenous |
| MAP | Mean Arterial Pressure |
| PA | Pulmonary Artery |
| PFC | Perfluorocarbon |
| $P_{ISF}O_2$ | Interstitial oxygen tension |
| PQ | Phosphorescence Quenching |
| SEM | Standard Error of the Mean |
| $SpO_2$ | Oxygen Saturation |
| $SvO_2$ | Mixed venous oxygen saturation |
| T(n) | Time in *n* minutes after onset of resuscitation |