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14. ABSTRACT (around 200 words)

Perfluorocarbon emulsions (PFCs) can treat traumatic injuries (traumatic brain injury (TBI), hemorrhagic shock and burns by enhanced delivery of oxygen. A class-based side effect of PFC (day 2-5 after infusion in 30-50%) may be thrombocytopenia (TCYP). The mechanism is inadequately investigated. The US Food and Drug Administration (FDA) requests investigation of the phenomenon to exclude platelet inflammatory/embolic safety risks. The initial results (phase I) showed that PFC infusion in the normal sheep did not significantly change the platelet number and activation among the experimental and control groups. In 2015 (Phase II & III), PFC infusion as a part of resuscitation fluid was used in sheep with hemorrhage (n=39) and polytrauma (blast traumatic injury and hemorrhage, n=8). The results showed that the sheep's platelet count and fibrinogen level were reduced immediately after resuscitation. However, there was no significant change of platelet number and activation after PFC infusion compared with non-PFC controls over the 7 survival days. Platelet contractile force (PCF, Platelet activator) also showed no significant change compared with control groups (saline & surgical control). Platelet morphological observation corresponded well with function assays. There were no significant percentage changes in neutrophils and monocytes after PFC infusion in injured sheep.

15. SUBJECT TERMS
Platelets, Perfluorocarbon emulsions (PFCs), Sheep, Coagulation, Hemorrhage, Resuscitation

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INTRODUCTION

Perfluorocarbon emulsions (PFCs) are a small volume robust (temperature stable, long storage life, portable) intravenous (i.v.) fluid, easily carried by medics/corpsmen to site of first contact. PFCs enhance O_2 solubility/diffusion from circulating red cells. PFCs have shown efficacy in animal models of hemorrhagic shock, tissue ischemia, decompression sickness (DCS), traumatic brain injury (TBI) and other important military applications. Our work and that of others demonstrated that PFCs enhance O_2 delivery at normal FiO_2 and that perhaps the most important aspect of PFC infusion was an enhanced O_2 delivery from native erythrocytes to tissues. Furthermore, it appears that PFCs enhance O_2 diffusion, thereby decreasing the barrier to non-polar gas movement made up of aqueous materials (plasma and extracellular fluids). However, a class-based side effect of PFC (day 2-5 after infusion in 30-50%) is thrombocytopenia (TCYP). The mechanism is inadequately investigated but is caused by reduced production or enhanced clearance (partial activation) of platelets (Plts). These safety concerns posed by the United States Food and Drug Administration (FDA) have to do with a potential risk of hemorrhage/thrombosis and inflammation related to PFC infusion. Casualty care for hemorrhage, gas embolism (blast and DCS) and TBI all involve degrees of inflammatory up-regulation and variable elements of coagulopathy. The current approved work is to answer safety and mechanism questions regarding causes/extent of thrombocytopenia after PFC infusion. Pertinent large animal models of normal and casualty scenarios will be investigated, thereby demonstrating whether the use of PFC in hemorrhage and blast TBI possess any added coagulopathic risk to future victims, compared to normal. Large animal models will examine specific causal hypotheses for TCYP and whether this exists as a class effect. In the end, the work will provide answers to questions blocking further development of PFCs. In this proposed study, the side effects of two PFC's on platelet count, structure and function will be tested. **PHER-O_2** or **Perftoran** contains perfluorodecalin (88% or 20% w/v, respectively), purified water and an emulsifier that allows the product to be administered intravenously. Perfluorodecalin is a biologically inert substance that is not metabolized by the body but rather is excreted from the body through normal respiration. **Oxygent**, another resuscitation product, contains perflubron emulsion (60%, w/v) and has a similar O_2 carrying capacity like PHER-O_2. In the present study, the specific aims are to answer the following: #1 Whether PFC infusion activates Plts in vivo, #2 Whether Plt/white cells clumps (microaggregates) occur, and #3 Evaluate the mechanisms of partial Plt activation (if it occurs).

BODY OF REPORT

Material and Methods:

All animals (sheep) received humane care in compliance with the “Eighth Guide for Care and Use of Laboratory Animals”, prepared by the National Academy of Sciences and published by the National Institutes of Health. This study was approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) certified Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC) and was also approved by the USAMRMC Animal Care and Use Review Office (ACURO).

Study Design:

Year one: (completed) A normal sheep (ovine, 20-30 kg) model to test the effect of PFC intravenous infusion on platelet number and activation was used. Sheep were randomly divided into 4 groups (Oxygent, Perftoran, hetastarch and saline/naïve groups, n=8/each group). Venous blood samples were collected at baseline, 0 minute after PFC infusion, 3, 24, 96 hours and 7 days post PFC infusion for

Plt/white cell activation (Plt number, Plt white cell aggregates, flow cytometry-glycoprotein expression), other coagulation data, (RoTEM, Platelet Shear Modulus, PFA-100 and Plt aggregometry) and compliment expression. Samples were also examined with scanning electron microscopy for Plt activation morphology.

Year two: A sheep (ovine, 20-30 kg) hemorrhagic shock model was utilized to test the effect of PFC intravenous infusion on platelet number and activation. Animals were anesthetized, instrumented, bled (35~50% total blood volume) and mean arterial pressure maintained at 30 mmHg (±3 mmHg) for 60 minutes. Animals were then resuscitated with hetastarch plus PFC (oxygent, n=6) or hetastarch plus saline (n=7). There was also a surgical control group (n=6). Venous blood samples were collected at baseline, 1 hour after PFC infusion, 24, 96 hours and 7 days post PFC infusion for Plt/white cell activation (Plt number, Plt white cell aggregates, flow cytometry-glycoprotein expression), other coagulation data (RoTEM, Platelet Shear Modulus, PFA-100 and Plt aggregometry) and compliment expression. Samples were also examined with scanning electron microscopy for Plt activation morphology.

Year three: An ovine polytrauma model of blast traumatic injury followed by hemorrhagic shock will be used to test the effect of PFC intravenous infusion on platelet number and activation. Volume resuscitation will occur with either hetastarch and saline or hetastarch and PFC. Similar studies of Plt and white cell activation will be carried out.

Subjects: When Juvenile sheep (Dorset/Dorper cross, 25-30 kg) were shipped to VCU DAR facility, general health checkup was taken immediately by a veterinarian, including measurements of sheep body temperature, heart rate and respiratory auscultation. Venous blood samples were drawn for complete blood count (CBC). Stool samples were examined for any parasite infections. Sheep were acclimated for 7 days in order to recover from shipping fever or to treat any potential infection. Sheep were randomized into different groups (see above study design, year one and two). In 2015, 37 total animals were ordered and used for 41 different experiments (control animals were reused 7 days after recovering from top-load blood collections). The following chart describes the animal usage in detail for 2015.

2015 Animal use

Quarter Period	Sheep ordered	Top-load model	Hemorrhagic shock	Polytrauma (BTBI+HS)	Sum of Used	Model development /death
I	**11**	**0**	**11**	**0**	**11**	**2**
II	**8**	**4**	**8**	**0**	**12**	**4**
III	**10**	**0**	**8**	**2**	**10**	**2****
IV	**8**	**0**	**0**	**8**	**8**	**0**
Total	**37**	**4***	**27**	**10**	**41**	**8**

Note: 1. * 4 animals were used as naïve control and re-used after one week recovery for hemorrhagic shock study in order to reduce animal use.
2. **Model development was for polytrauma model which is combined air blast traumatic injury plus hemorrhagic shock and also for DAR veterinarian observation.
3. Total 6 sheep were euthanized after hemorrhagic shock resuscitation due to the weakness based on the advices of our veterinarians.

Animal procedures:

1. Top-load Study: Coagulopathy in sheep top loaded with PFC or hetastarch was assessed at baseline prior to compound administration and at time zero, 3 hours, 1 day, 4 days, and 7 days following infusion. Baseline venous samples via external jugular vein puncture were taken two days before top-load experiments. Sheep were fasted for 24 hours before the procedure. On procedure day, sheep were anesthetized with 4~5% isoflurane via vaporizer cart. Once unconscious, anesthesia was maintained with 2~3% isoflurane based on the anesthesia level assessment. Animals were transported to the laboratory. Then, the animals were intubated and ventilated with 70% nitrogen/30% oxygen mixture. The animal's neck area was shaved and disinfected with 70% ethanol and betadine as well as covered with a surgical drape. Local lidocaine was used to reduce pain. A jugular needle catheter (20 Gauge, 2 inch in length) was placed for PFC or Hespan infusion (3g/kg) over 15 minutes. Immediately following infusion, time zero blood sample was collected. The jugular catheter was removed and the puncture site sanitized. The initial top-load procedure was about 20~40 minutes. There was no dehydration during this short period. During the procedure, body temperature was maintained with a pre-warmed heating blanket. Animal's heart rate and oxygen saturation were monitored. The animal was then transported back to DAR vivarium for recovery from anesthesia. Animals were monitored and weighed on a daily basis to ensure proper food intake and hydration. Note that animal venous blood was sampled via external jugular vein puncture for baseline, 3 hours after top-load, 24 hours, 4 days and 7 days post top-load without anesthesia. Blood sampling without anesthesia is a common veterinary practice and minimizes respiratory distress and the potential for decreased food intake and dehydration from repetitive daily exposure to gas anesthesia.

2. Hemorrhagic Shock Study: Sheep were handled in a similar way as the top-load study. Baseline venous samples taken two days before the experiments. Sheep were fasted for 24 ~ 48 hours before the procedure. On procedure day, sheep were anesthetized with 4~5% isoflurane via vaporizer cart. Once unconscious, anesthesia was maintained with 2~3% isoflurane based on the anesthesia level assessment. Animals were transported to the laboratory. Then, the animals were intubated and ventilated with 70% nitrogen/30% oxygen. The animal's neck area was shaved and disinfected with 70% ethanol and betadine as well as covered with a surgical drape. Local lidocaine was used to reduce pain. A central jugular line was placed for blood sampling and resuscitation. Both sides of femoral arteries and veins were cut-down near the tip of femoral triangle distal to major branches and catheters were placed. The right femoral artery was cannulated with a PE-240 catheter for hemorrhage; the right femoral vein was cannulated with PE190 catheter for blood sampling and fluid resuscitation. The left femoral artery was cannulated with a PE-90 catheter for continuous blood pressure monitoring and the left femoral vein was cannulated with Swan-Ganz for pulmonary arterial pressure and cardiac output monitoring as well as mixed venous blood sampling. All vital parameters were continuously monitored with Biopac data acquisition system (www.biopac.com). Arterial and mixed venous blood samples were collected every 20 minutes during hemorrhage and resuscitation period. Animals were stabilized for 10 minutes after all surgical procedures were completed. A three-stage hemorrhagic shock model was used (see attached flow chart for details). Total average amount of blood loss was 32% ~ 50% and the mean arterial pressure (MAP) was maintained at 30±3 mmHg for 60 minutes followed by fluid resuscitation. All hemorrhagic animals were resuscitated with intravenous hespan (hetastarch) first until MAP reached 65 mmHg and stabilized for 10 minutes followed by intravenous infusion with PFC (Oxygent, 60%, 3g/kg;

Perftoran, 20%, 1g/kg) over 15 minutes or the same amount saline. Animals were closely monitored for 60 minutes before being recovered from anesthesia and moved back to the DAR facilities. Blood samples were collected at 60 minutes post resuscitation, 24 hours, 4 days and 7 days for coagulopathy analysis. The hemorrhagic animals transported back to DAR vivarium were monitored on a daily basis to ensure proper food intake and hydration. Due to the severity of the hemorrhage model, the death rate for the current hemorrhagic sheep model is approximately 15 ~25%.

3. Polytrauma Model Study: Sheep will be fasted for 24 hours prior to each blast to minimize aspiration of stomach contents. Sheep (18~32kg) will be initially anesthetized with 5% isoflurane. Once anesthetized, animals will be intubated with an endotracheal tube (ID= 9~10 mm with cuff), an orogastric tube placed to expel vomit, and ventilated with mixed nitrogen/oxygen (80:20). Anesthesia will be maintained with 1.5~2% isoflurane and the vaporizer driven with medical air. A percutaneous lumbar puncture will be performed in order to sample the baseline CSF. A dose of carprofen (3mg/kg, i.m., effective for 24 hours) will be given during the surgery procedure to reduce pain. Animals, still anesthetized, will then be placed in a prone position on a surgical tray with the head placed in a stereotaxic frame for the blast wave exposure. The animal will be placed in the test section of the Advanced Blast Simulator with head facing the oncoming overpressure wave and subjected to a single shock wave of 15-25 psi (see attachment of blast device). Following exposure to blast, sheep will undergo the hemorrhagic shock protocol as described above starting within 15 minutes. Sheep will then randomly be assigned to receive either PFC (Perftoran) or saline infusion after initial resuscitated with hetastarch. Sheep will be recovered from anesthesia and will be returned to its flock (as described as in hemorrhagic model study).

Study endpoints:

1. **Blood sample analyses** including **coagulopathy tests** (platelet number and activation, same protocol as reported in 2013); **blood biochemistry** and **platelet morphologic observation** using scanning electron microscopy (same protocol as reported in 2014) will be examined. Also, **white blood cell counts** especially neutrophils and monocytes will be analyzed to reveal any correlation with changes of coagulation after hemorrhagic shock over 7 days.

2. **Hemorrhagic Physiology** including monitoring blood pressure, heart rate, ECG, central venous pressure, pulmonary arterial pressure, SvO_2, cardiac output and blood gas analysis during the hemorrhagic shock and resuscitation will be studied.

3. Sheep behavioral monitoring is entirely observational and the sheep are in their own enclosure with the rest of their flock during the period of observation. Video cameras are used to monitor the sheep 24/7 before and after experiments. Scoring of the video records is done by an observer who is blind to the treatment status of the sheep in question and are scored based on the proportion of each day that the sheep spends actively moving around the enclosure, feeding, or lying down and inactive. After the conclusion of the experiment all animals are humanely euthanized. Sheep are video monitored from 2 days before top load / hemorrhage / polytrauam through 7 days after the experiment. Screen monitoring and video record materials are protected and accessed only by authorized personnel following IACUC guide lines.

4. **Biomarker analysis in hemorrhagic shock and polytrauma injury models** include measuring

Alpha 2 spectrin and S-100β protein markers in venous blood and cerebrospinal fluid samples.

Statistical analysis:

Power analysis based on sheep platelet mean number was used to estimate animal numbers per experimental group. JMP pro 12.0 statistical software was used to analyze all blood sample results. Data distribution and one-way analysis of variance (ANOVA) were used to compare means. Data were compared among groups and within the group at different time points. Significant difference between means was p value less than 0.05 ($p<0.05$).

RESULTS

Results (sheep behavioral monitoring)

All sheep subjected to blood sample analyses outlined above were observed behaviorally using non-interfering video camera to hard drive recording from 2 days prior to experimental procedure through the duration of the blood sample time points. Data are currently being analyzed and will be presented in future reports.

Result summary:

1. In the current study period (2015), 36 healthy sheep received intravenous infusion of PFC (oxygent and perforan) (total 32 animals, 4 groups with n=8 animals per group; plus 4 control animals were added in 2015; phase I). PFC animals showed no significant reduction of platelet count nor revealed significant activation of platelets when compared with control groups. Three full publications are being prepared (see working publication title list).

2. In the current study period (2015), the PFC (Oxygent and Perftoran) infusion as a part of resuscitation fluid in survival sheep hemorrhagic shock model was completed (total 39 animals, phase II). Some of the data was presented in August 2015 at the Military Health System Research Symposium (MHSRS) and VCU student research day in May 2015. Some hemodynamic monitoring data will be presented at the 2016 annual meeting of International Anesthesia Research Society (IARS) (see attachment for the posters and abstract). Venous plasma S-100β and alpha 2 spectrin expression analyses were completed and showed no significant difference among PFC groups versus non PFC control groups. A total of 27 animals were completed in this period and 21 survived for 7 days after hemorrhagic procedures. Initial data analysis showed that PFC infusion after hemorrhagic shock did not cause further decrease in platelet count nor change of its activation when compared with non-PFC group or surgical control group. More detailed data analysis and publication are ongoing.

3. In the current study period (2015), the polytrauma injury model development which combines blast trauma injury followed by hemorrhagic shock was completed. The study of PFC (perftoran) infusion as a partial resuscitation fluid after polytrauma injury is ongoing (phase 3). A total of 10 animals were used for the study, in which 2 animals were used for model development and veterinarian observation as a part of animal use protocol compliance required by VCU IACUC.

Another 8 animals were randomly assigned into PFC group (n=3) or control group (n=5). Animal experiments will be completed in the first quarter of 2016 (n=20) during the no-cost extension period. Platelet number measurement and functional assays are ongoing and the data base is being continually updated. Data analysis will start when experimental animals and blood sample analysis are completed.

PROBLEMS AND SOLUTIONS

1. At the beginning of the year, although we had moved the laboratories back to the renovated spaces in October 2014, the biochemistry laboratory was not completely set up until the second quarter period. **Solution:** Animal experiments were on schedule and blood samples were collected. Blood sample analysis for biochemical marker measurement were caught up to date.

2. Characterization of the Advanced Blast Simulator for the development of the blast TBI / hemorrhage polytrauma model was delayed due to flooding of laboratory space at the end of 2013. Advanced Blast Simulator setup was completed and passed veterinarian observation in the third period of this year. **Solution**: We will expedite experiments and catch back up to the study timeline.

3. We have run out of one of the PFCs (Oxygent) and an order was placed in April but we are still waiting for product shipment. **Solution**: Keep contacting the vendor asking to ship the product as soon as possible and looking for any new vendors who can provide medical grade PFC.

4. Platelet functional assay: there were a few measurements of data which drifted away from baseline without clear reason (might be the size of sheep platelet is smaller than human's, the analyzer over counting). Some of the assays are still waiting for analysis until large enough sample size is obtained. **Solution**: Sample values were doubled and repeatedly measured. Statistician is involved in data analysis to make sure all data are analyzed using correct methods.

5. Due to the unexpected flooding of the laboratories in November of 2013 and because we had to relocate laboratories twice over the last 2 years, the study is behind. The completion of the animal study is expected by April 1, 2016 and the whole funded program is expected to complete by June 30, 2016. **Solution:** We have filed and were granted a no-cost extension for this project.

KEY RESEARCH ACCOMPLISHMENTS

1. One first year medical student was awarded a medical student summer research fellowship based on this study. The work will be presented in May, 2016.

2. Based on phase II results, an abstract was submitted and accepted by MHSRS as a poster presentation in August 2015. A poster was presented on student research honor day (Medical student summer research presentation on May 1, 2015).

4. Initial phase II study data analysis showed that intravenous PFC infusion after fluid resuscitation in hemorrhagic sheep did not cause further reduction of platelet number and did not significantly change platelet activation when compared with non-PFC groups.

4. A survival polytrauma model has been established in which sheep are subjected to blast trauma injury followed by hemorrhagic shock (study phase III). Animal experiments will be completed in the first quarter of 2016.

5. Plasma alpha 2 spectrin and S-100β (apoptotic/necrotic neuronal death and blood-brain barrier disruption, respectively) analysis in hemorrhagic sheep was completed. Initial data analysis did not show a significant change among experimental and control groups.

6. Manuscripts for phase I and II studies and abstracts for 2016 MHSRS are in progress based on the results of the current study.

7. Current study budget partially supports 4 full time employees and two part time employees.

REPORTABLE OUTCOMES

1. Effect of PFC infusion on platelet number and function in the healthy sheep portion of the study is being completed. Publications are being prepared (Total 32 animals in 4 groups. Three papers are being written).

2. Effect of intravenous PFC on platelet number and function in the survival hemorrhagic sheep portion of study has been completed (Total 39 animals in 4 groups). Two abstracts have been presented in 2015 and one poster will be presented in 2016. Full data analysis and publications are being prepared.

3. A polytrauma injury model combining blast injury first followed by hemorrhagic shock has been established and Phase III study of the funded program is ongoing. The animal experiments will be completed in the first quarter of 2016.

4. Based on the data analyses from phase I and II studies, the results show that intravenous PFC infusion in healthy and hemorrhagic shock animals did not result in severe thrombocytopenia or coagulopathy compared with non-PFC control groups. These data are encouraging for FDA approval of further clinical trial study of PFC in the United States.

CONCLUSION

1. Intravenous PFC infusion in healthy sheep or hemorrhagic sheep did not significantly reduce platelet number nor significantly alter platelet function based on the current research data.

2. These results suggest that further study of PFC is warranted as planned. This research project going forward will assess PFC's effect on platelet number and function in sheep polytrauma model which combines blast head injury and hemorrhagic shock.

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APPENDICES

1. Publications on preparing:

1) Effect of Perfluorocarbon Infusion on Platelet Number in Normal Male Juvenile Sheep
2) Effect of Perfluorocarbon Infusion on Platelet Activation in Normal Male Juvenile Sheep: Scanning and Transmission Electronic Microscopic Analysis
3) Effect of Perfluorocarbon Infusion on Platelet Activation in Normal Male Juvenile Sheep: ROTEM Assay Analysis
4) Effect of Perfluorocarbon Infusion as A Part of Resuscitation Fluid on Platelet Number and Activation in Hemorrhagic Sheep
5) Evaluation of Noninvasive Cardiac Output Monitoring in Sheep with Hemodynamic Instability

2. **Abstracts/posters were presented in 2005 and submitted for 2016**
   1) 2016 International Anesthesia Research Society Annual meeting (May 19-24, 2016): Paul A. Middleton, MD, Penny S. Reynolds, PhD, Jiepei Zhu, MD PhD, Bruce D. Spiess, MD. ***Evaluation of Noninvasive Cardiac Output Monitoring in Sheep with Hemodynamic Instability*** (submitted)

   2) 2015 MHSRS (Military Health System Research Symposium, August 17-21, 2015: Jiepei Zhu[1,4], J. Travis Parsons[2,4], Erika J. Martin[3], Jacquelyn R. McCarter[2], Christopher R. Sweeney[1], Paul A. Middleton[1], Brian E. Berger[1], Evan J. Kraus[1], Donald Brophy[3,4] and Bruce D. Spiess[1,4] ***Effect of Intravenous Perfluorocarbon on Platelet Number and Function in Hemorrhagic Sheep*** (MHSRS-15-0376)

   3) 2015 MHSRS (Military Health System Research Symposium, August 17-21, 2015: Matt Hallman[1], Kyle Flores[1], Jacquelyn McCarter[2], Chris Sweeney[3], Andrew Morris[3], Jiepei Zhu, PhD, MD[3,4], Bruce Spiess, MD[3,4], J. Travis Parsons, PhD[2,4] ***The Effect of Perfluorocarbon Oxygen Therapeutics in a Sheep Survival Model of Severe Hemorrhagic Shock*** (MHSRS-15-2041)

   4) 2015 Virginia Commonwealth University, Student Research Honor Day (May 1, 2015): Evan Kraus*, Jiepei Zhu[1,4], J. Travis. Parsons[2,4], Erika J. Martin[3,4], Jacquelyn McCarter[2], Christopher Sweeney[1], Paul Middleton[1], Donald Brophy[3,4], Bruce D. Spiess[1,3,4], ***Effect of Intravenous Perfluorocarbon on Platelet Number and Function in Hemorrhagic Sheep***. (* First year medical student, Dean's research fellowship winner)

3. **Blast trauma injury device and polytrauma model with Biomarker results**

4. **Summary Data sheet – PFC infusion as a part of resuscitation fluid in hemorrhagic sheep**

5. **Initial summary Date sheet - PFC infusion as a part of resuscitation fluid in Polytrauma sheep combined blast trauma injury following hemorrhagic shock**

Effect of Intravenous Perfluorocarbon on Platelet Number and Function in Hemorrhagic Sheep

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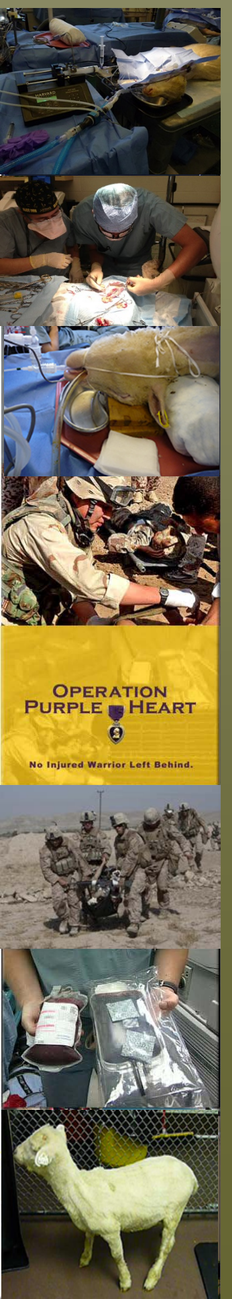


Introduction

PFC is a non-polar oil/emulsion with enhanced respiratory gas (O_2, N_2, CO_2) solubility found in 1966. All O_2 dissolved in PFC is available for metabolic use, which is called an O_2 carrier. PFC particles are 0.1~0.2 µm and get into tissues where RBCs cannot after injury. PFC as an extra compartment for O_2 transport and has a unique efficacy in low flow states. PFC has shown efficacy in models (some human data) of hemorrhagic shock, traumatic brain injury (TBI), spinal cord injury, decompression sickness (DCS), arterial/venous gas embolism (A/VGE), and can be used as a part of a battlefield intravenous resuscitation fluid by enhanced oxygen delivery for trauma hemorrhagic injury and en route care.

A possible side effect of PFC might be related with thrombocytopenia (in 30~50%) on days 2~5 after intravenous infusion. It is necessary to investigate this phenomenon to exclude platelet inflammatory/embolic safety risks before clinical trials resume. Using a sheep hemorrhagic shock model to investigate the effect of PFC on sheep platelet number and function.

Materials & Methods

Subjects and Groups:

- The experimental protocol was reviewed and approved by the Animal Care and Use Committee of Virginia Commonwealth University
- Total 27Juvenile Dorset or Dorper sheep (18-32 kg body weight) were survived through the study. Animals were given 7 days for acclimation prior to experiment, and daily vital signs are monitored including temperature, heart rate and respiratory rate. 18 hemorrhagic shock sheep were randomly resuscitated with hespan plus PFC (Oxygent, 5 ml/kg, n=9) or resuscitated with hespan plus saline (5ml/kg, n=9). 9 sheep were used for surgical sham control group.

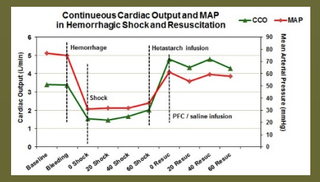
Hemorrhagic Shock and Resuscitation Procedures & Blood Sample Collection:

- Animal was induction of anesthesia with 5% isoflurane and maintained with 1~2% isoflurane during the procedure.
- Animal was intubated (7.5 F) and ventilated (Drager Fabius GS Anesthesia Machine) with 30% O_2: 70% N_2)
- Animal's femoral arteries and veins were placed with catheters including a swan ganz for hemodynamic monitoring and resuscitation. Hemodynamic monitoring include arterial blood pressure (BP) and mean arterial blood pressure (MAP); Central venous blood pressure (CVP); Pulmonary arterial blood pressure (PAP); Cardiac output and arterial, venous blood gases.
- A survival moderate stepwise hemorrhagic shock model in sheep: bleeding 35~50% total blood volume, MAP=30 mmHg for 60 minutes (class III shock model). The first fast bleeding phase: 3ml/kg/min till MAP=45 mmHg; The second bleeding phase: 2 ml/kg/min till MAP =40 mmHg starting at MAP recovered to 55 mmHg or 15 minutes after the first fast bleeding. The third bleeding phase: 1 ml/kg/min till MAP =30 mmHg (±3mmHg) starting at MAP recovered to 45 mmHg or 15 minutes after the second bleeding.
- Resuscitation was first intravenously infusion hespan (6% hetastarch) till MAP = 65 mmHg. Then, animal was stabilized for 10 minutes and maintain MAP =55 mmHg before added PFC or saline (5 ml/kg) infusion for 15 minutes. Animal was observed for 60 minutes after resuscitation completed. Then, animal was allowed to recover from anesthesia and carried back to DAR facility.
- Venous blood was collected via jugular vein puncture at baseline, 1 & 24 hour and 4 & 7 day post shock.

Experimental timeline

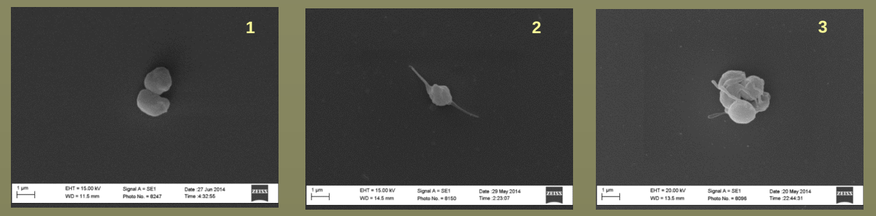
Blood sample measurement & Data analysis:

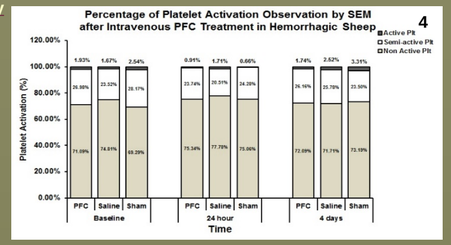
- Venous samples were measured for coagulatory factors including:
  Platelet number count (VetScan HM5 Hematology system); Fibrinogen (Diagnostica Stago analyzer).
  Platelet contractile force (PCF); Clot elastic modulus (CEM); with Rotem® *delta* (Native, Intrinsic, Extrinsic).
  Collagen Aggregation, ADP aggregation were measured with 700 Aggregometer.
- Venous samples collected at baseline, 24 hour and 4 days post shock were processed for scanning electron microscope observation.



Results

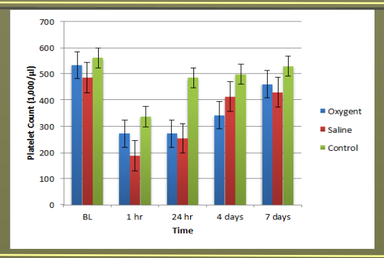
Morphology of platelet observation with scanning electron microscopy

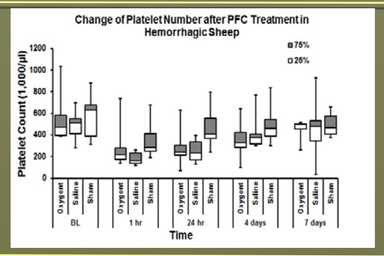




Criteria Used to Analyze Platelet Count Images

Figure 1: (left) Non-active platelets: Non-active platelets are small size with smooth surface. **Figure 2:** (middle) Semi-active platelets are with one or 2 processes.
Figure 3: (right) Active platelets are with 3 or more processes (Pseudopods) and their surface becomes irregular or granular. Or conjugated platelets which groups of platelets that have pseudopods connected
Figure 4. Percentage distribution of non-active, semi-active and active platelets .

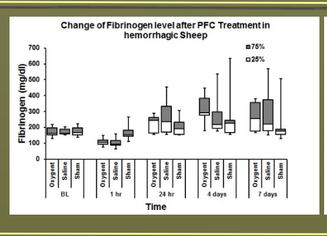


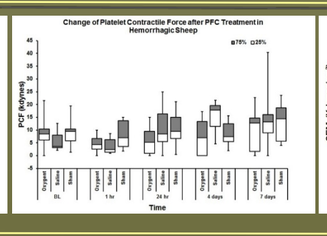


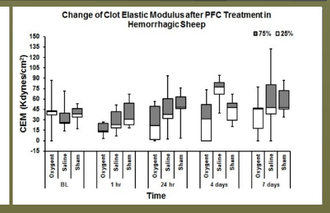
Platelet number: In normal sheep, platelet mean value is about 400,000/µl (range from 100,000 to 800,000). Our data showed that platelet count was significantly decreased at 24 hour after PFC or saline resuscitation compared with the sham procedure group or with their baseline. But there was no significant change in platelet number between PFC and saline groups at 24 hours post resuscitation. There was no significant difference in platelet count at 4 days and 7 days post shock among the groups. Platelet count returned back to baseline level at 7 days after shock.

Left Figure: Platelet count and mean distribution with standard error.

Right figure: Box plot shows platelet count distribution with minimum value, maximum value, 25%, median, and 75% values





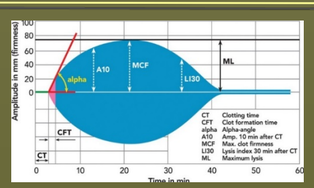


Fibrinogen measurement (left figure): analysis showed that fibrinogen dropped at one hour post resuscitation possibly because of hemodilution when comparing with sham group and baseline. There is no significant difference when the groups are compared each other at 24 hours and 4 & 7 days indicating no massive clot formation.

Platelet Contractile Force (PCF, middle figure): there is no significant difference in fibrinogen counts between the 3 groups though trend to decrease. PCF measures platelet function and is affected by platelet metabolic status, GP IIb/IIIa status, and presence of antithrombin activity.

Clot elastic modulus (CEM, on right figure) measures showed a trend decrease at 1 hour and 24 hour after PFC infusion comparing its baseline or sham. platelet function, changing with production of force by platelets, rate of thrombin generation, and platelet concentration.

PFC does not over activate or significantly reduce platelet function based on PCF & CEM measurements. Box plot figures showed the measurement distribution with minimum value, maximum value (error bar), 25%, median (between two boxes) and 75% values.



Rotational thromboelastometry (ROTEM) for hemostasis analysis

Table 1. Clotting Time (CT, seconds, mean ± SE)

Parameters	Groups	Baseline	1 hour	24 hour	4 days	7 days
Clotting Time (CT) INTEM (sec)	Oxygent	159.78±12.61	187.33±12.08	177.78±13.94	156.89±13.25	171.89±13.94
	Saline	160.44±9.01	180.22±7.87	147.78±10.86	138.78±4.72	162.00±10.86
	Sham	156.10±7.95	156.10±11.34	163.30±9.44	159.80±6.82	149.30±9.44
Clotting Time (CT) EXTEM (sec)	Oxygent	74.44±5.83	94.00±5.58	68.78±8.83	78.22±9.71	78.11±8.33
	Saline	68.44±5.22	89.00±6.09	75.78±9.24	69.89±10.00	78.00±9.24
	Sham	62.80±6.22	66.10±7.09	71.40±9.57	83.30±10.71	79.80±9.57

Clotting time comparison at baseline and various survival time points at post hemorrhagic shock. Adding PFC did not cause a significant longer clotting time.

Table 2. Maximum Clot Firmness (MCF, mm, mean ± SE)

Parameters	Groups	Baseline	1 hour	24 hour	4 days	7 days
Maximum Clot Firmness (MCF) INTEM (mm)	Oxygent	75.89±1.22	66.89±1.74	77.22±1.01	81.67±0.93	83.44±1.36
	Saline	76.00±1.09	66.78±1.26	75.44±1.39	81.33±1.42	83.44±1.39
	Sham	78.40±0.88	76.50±1.02	79.90±1.55	80.20±1.31	80.20±1.55
Maximum Clot Firmness (MCF) EXTEM (mm)	Oxygent	75.89±1.21	66.78±1.27	76.33±1.52	82.11±0.95	83.22±1.52
	Saline	76.00±1.00	66.89±2.85	72.67±1.54	81.22±1.47	83.89±1.54
	Sham	78.10±0.84	76.40±0.87	79.80±1.81	80.10±1.39	80.50±1.81

Maximum clot firmness comparison at baseline and various survival time points at post hemorrhagic shock. Adding PFC did not significantly reduce MCF.

Conclusion:

- After intravenous infusion oxygent (PFC) following shock, there is no significant change in platelet number, morphology and function comparing with saline control group.
- The result of quantitative observation of platelet is corresponded with the results of coagulation factor analysis.
- Therefore, intravenous infusion with Oxygent will not significantly worse the coagulopathy caused by hemorrhage.

Acknowledgements:

- The Microscopy Core Facility of VCU
- The work is funded by U.S. Army Medical Research and Materiel Command (W81XWH-13-1-0017)
- PI: Dr. Bruce Spiess

The Effect of Perfluorocarbon Oxygen Therapeutics in a Sheep Survival Model of Severe Hemorrhagic Shock

Matt Hallman[1], Kyle Flores[1], Jacquelyn McCarter[2], Chris Sweeney[3], Andrew Morris[3], Jiepei Zhu, PhD, MD[3,4], Bruce Spiess, MD[3,4], J. Travis Parsons, PhD[2,4]

School of Medicine[1], Department of Neurosurgery[2], Department of Anesthesiology[3], VCU Johnson Center for Critical Care and Pulmonary Research[4]
Virginia Commonwealth University, Richmond, Virginia, USA

MHSRS 2015 #2041

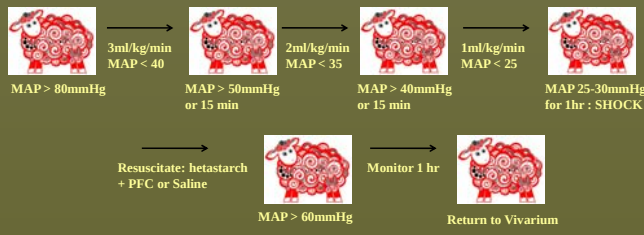
BACKGROUND

Severe hemorrhagic shock in the far forward battlefield can be life threatening with the absence of blood products and significant delay to higher echelon treatment facilities. Perfluorocarbon (PFC) oxygen therapeutics are capable of effectively oxygenating sensitive tissue in the absence of adequate hemoglobin and/or blood flow. PFC emulsion volumes required for efficacy can be readily carried in a medic pack and can be easily administered by minimally trained personnel. PFC may improve the "golden hour" during en route care of far forward battlefield polytrauma soldiers.

METHODS

Hemorrhagic Shock

Male sheep (20-30kg) were anesthetized, intubated, and ventilated on room air with 1-2% isoflurane. Animals were instrumented for measurement of vitals, hemodynamics, and sampling of blood for gases, biochemistry, and hematologic evaluation. Arterial blood was removed 3ml/kg/min until MAP below 40mmHg. Once MAP returned to 50 (or 15min), blood was removed 2ml/kg/min until MAP below 35mmHg. Once MAP returned to 40 (or 15min), blood was removed 1ml/kg/min until MAP below 25mmHg. Sheep remained at MAP 25-30mmHg for 1hr. Sheep were then resuscitated with minimal volume hetastarch (until MAP 60mmHg) then given either 6cc/kg saline (n=3) or Oxygent PFC (n=3). Following resuscitation, sheep vitals and hemodynamics were monitored for 1 hour before returning to vivarium.

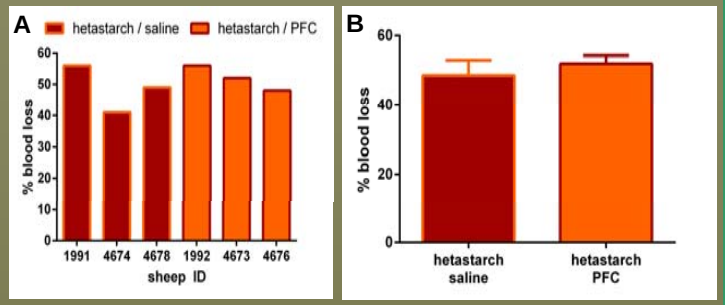


Behavioral Analyses

Efficacy of PFC treatment was evaluated by behavioral analysis using unobtrusive video camera monitoring of sheep in their social flock. Animals were considered injured if more time was required for return to ambulation and more time spent laying down than standing with the flock and eating.

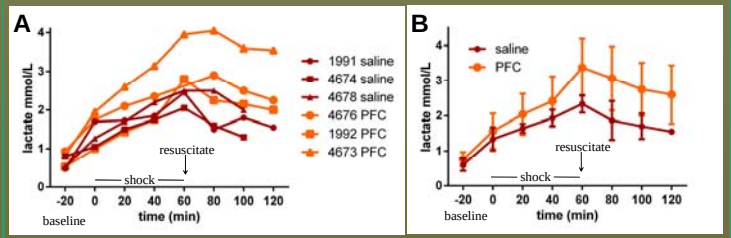
RESULTS

Total Blood Loss From Hemorrhage in (A) Individual Sheep and (B) averaged



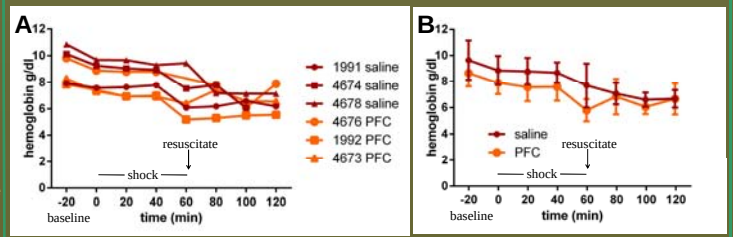
Total blood volume loss was 41-56% (48.7 ± 4.3 SEM) and 48-56% (52.0 ± 2.3 SEM) for shock saline and shock PFC treated, respectively

Lactate Levels in (A) Individual Sheep and (B) averaged



Lactate levels increased from baseline 0.60 ± 0.10 SEM and 0.76 ± 0.12 SEM mmol/L to maximal 2.33 ± 0.14 SEM and 3.38 ± 0.58 SEM mmol/L shock saline and shock PFC treated, respectively

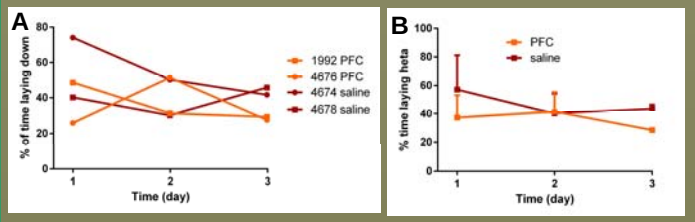
Hemoglobin Levels in (A) Individual Sheep and (B) averaged



Hemoglobin levels dropped from baseline 9.64 ± 0.87 SEM and 8.65 ± 0.60 SEM g/dL to minimal 6.61 ± 0.31 SEM and 5.80 ± 0.60 SEM g/dL shock saline and shock PFC treated, respectively.

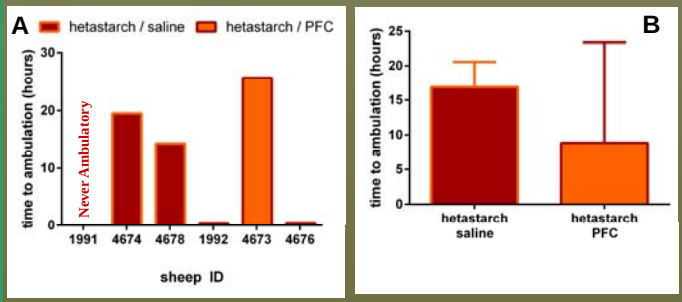
RESULTS (cont.)

Time Spent Laying Down in (A) Individual Sheep and (B) Averaged



Following hemorrhagic shock: on day one 57.20 ± 16.96% SEM vs 37.27 ± 11.26% SEM, on day two 40.25 ± 9.94% SEM vs 41.37 ± 9.92% SEM, and on day three 43.74 ± 2.03% SEM vs 28.55 ± 0.87% SEM of the time laying down, shock saline and shock PFC treated, respectively.

Time Spent Laying Down in (A) Individual Sheep and (B) Averaged



Shock saline animals took 14.3 and 19.5 hrs (16.90 ± 2.60 SEM) to ambulation and one never recovered. Shock PFC sheep took 25, 27min, and 25.6hrs (8.85 ± 8.41 SEM) to ambulation.

CONCLUSIONS

- In hemorrhagic shock animals with similar loss of blood, rise in lactate levels, and drop in hemoglobin values, the data suggest PFC treatment improves outcomes compared to animals treated with saline.
- This improvement occurs under normoxic conditions without the need for higher-than-atmospheric FiO2, not readily available in the far forward arena.
- PFC given acutely following minimal volume resuscitation after severe hemorrhage may improve outcomes for soldiers with significant evacuation timelines to higher echelon treatment facilities.

Acknowledgements: Supported by VCU Dept of Surgery Trauma Fund to JTP and U.S. Army Medical Research and Materiel Command (W81XWH-13-1-0017) to BDS

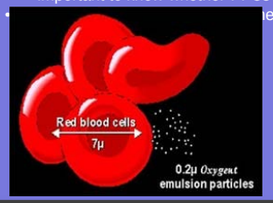
Effect of Intravenous Perfluorocarbon on Platelet Number and Function in Hemorrhagic Sheep

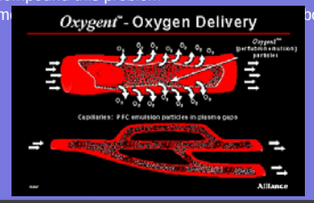
Evan Kraus, Jiepei Zhu[1,4], J. Travis. Parsons[2,4], Erika J. Martin[3,4], Jacquelyn McCarter[2], Christopher Sweeney[1], Paul Middleton[1], Donald Brophy[3,4], Bruce D. Spiess[1,3,4], M.D.,*

** MD candidate, Class of 2017, [1]Departments of Anesthesiology, [2]Neurosurgery, [3]Pharmacy, and [4]VCU Johnson Center for Critical Care and Pulmonary Research, School of Medicine, Virginia Commonwealth University, Richmond, VA 23298-0695*

Introduction

- PFC is a non-polar oil/emulsion with enhanced respiratory gas (O_2, N_2, CO_2) solubility found in 1966.
- PFC Particles are smaller than RBC and can carry oxygen around blood clots and into tissues where blood flow is restricted
- PFC emulsion increases brain tissue oxygen consumption in traumatic brain injury investigations
- It increased arterial oxygen concentration in a decompression sickness model
- PFC was shown in a baboon model to cause thrombocytopenia (30-50%), warranting further investigation
- Previous study showed no significant PFC effects on platelets count and activation following a topload without shock.
- PFC has high potential for use in hemorrhagic shock situations, where platelet may already be affected. It is important to know whether PFCs would compound this problem
- …e phenom… …olic risks





Materials & Methods

Subjects and Groups:

- The experimental protocol was reviewed and approved by the Animal Care and Use Committee of Virginia Commonwealth University
- Total of 27 Juvenile Dorset or Dormer sheep (18-32 kg body weight) were used and randomly divided into 3 groups: PFC hemorrhage group (n=9); Saline hemorrhage group (n=9); and Sham surgery group (n=9)

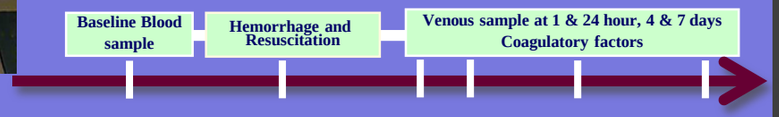
Hemorrhage Procedure & Blood Sample Collection:

- Animal was induced in anesthesia with 5% isoflurane and maintained with 2% isoflurane.
- Femoral cutdown performed bilaterally to draw blood, insert Swan-Ganz, and resuscitate as well as monitoring all physiological parameters: MAP, CVP, PA , CO, SVO2 etc.
- Sham surgery involved all of the above steps but not the hemorrhage or resuscitation.
- Sheep in hemorrhage groups had blood drawn in stepwise fashion until MAP was below 30 mmHg, maintained at that level (shock) for 60 minutes.
- Hespan resuscitation administered until sheep MAP maintained at 60 mmHg for 10 minutes.
- Either PFC (Oxygent, 60% v/w, 3g/kg or 5 ml/kg) or saline administered (5 ml/kg)
- Animal was allowed to recover from anesthesia and carried back to DAR facility after resuscitation completed.
- Venous blood was collected via jugular puncture immediately after resuscitation, 24 hours, and 4 & 7 days post trial

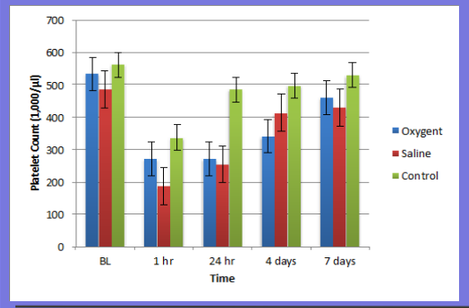
Blood sample measurement & Data analysis:

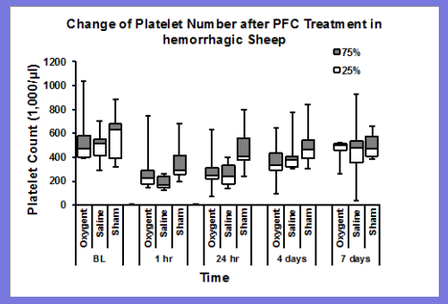
- Venous samples were measured for coagulatory factors including: platelet count, fibrinogen, Clot Elastic Modulus (CEM), and Platelet Contractile Force (PCF)
- Venous samples were collected at baseline, 1 hour, 24 hours, 96 hours, and 7 days after resuscitation

Experimental timeline



Results

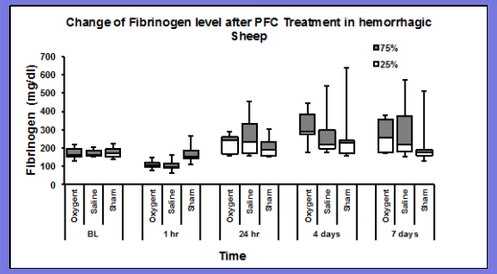




Platelet Number: In normal sheep, platelet mean value is about 400,000/μl. Our data showed that platelet count was significantly decreased at 24 hour after PFC or saline resuscitation compared with the sham procedure group or with their baseline. But there was no significant change in platelet number between PFC and saline groups at 24 hours post resuscitation. There was no significant difference in platelet count at 4 days and 7 days post shock among the groups. Platelet count returned back to baseline level at 7 days after shock..

Left Figure: Platelet count and mean distribution with standard error

Right figure: Box plot shows platelet count distribution with minimum value, maximum value, 25%, median, and 75% values

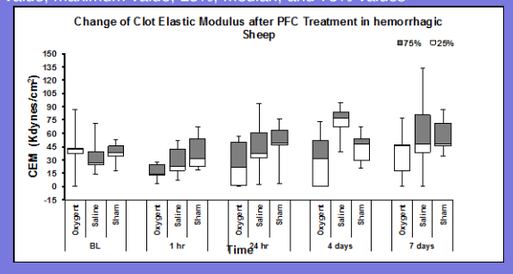


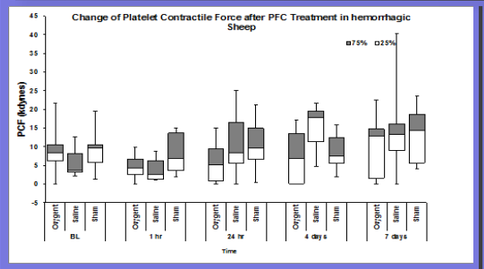
Fibrinogen measurement: there is no significant difference in fibrinogen counts between the 3 groups. There is also no significant change within each group, indicating no massive clot formation.

Clot elastic modulus: there is no significant difference in fibrinogen counts between the 3 groups

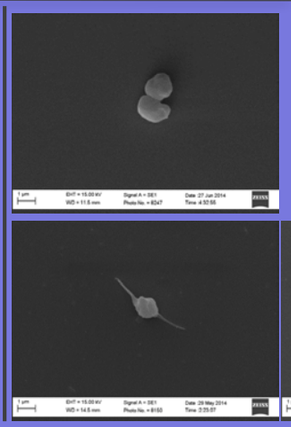
CEM measures platelet function, changing with production of force by platelets, rate of thrombin generation, and platelet concentration.

PFC does not over activate or significantly reduce platelet function based on CEM measurements.



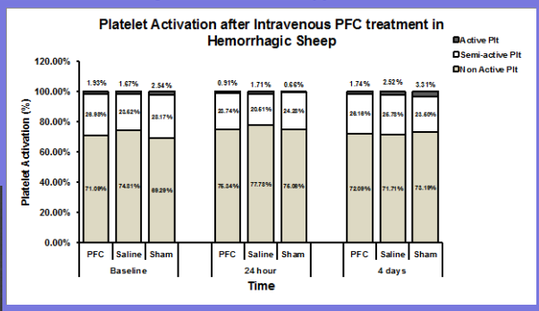


Platelet Contractile Force: there is no significant difference in fibrinogen counts between the 3 groups.
PCF measures platelet function and is affected by platelet metabolic status, GP IIb/IIIa status, and presence of antithrombin activity.
PFC does not over activate or significantly reduce platelet function based on PCF measurements.



Morphology of platelet observation with scanning electron microscopy

Top: non-activated platelets
Bottom left: semi-activated platelet
Bottom right: fully-activated platelets
Figure at right: There are no significant differences in platelet morphology between groups.



Criteria used to analyze platelet count images

- Semi activated platelet: 1 or 2 pseudopods
- Fully activated platelet: 3 or more pseudopods or conjugated platelets which are groups of platelets with pseudopods connected

Conclusion:

- After intravenous infusion PFC following shock, there is no significant change in platelet number, morphology, or function.
- In a sheep model, PFC does not cause significant effect on platelets

Acknowledgements:

- **VCU School of Medicine Summer Research Fellowship (Mentors: Drs. Bruce Spiess & Jiepei Zhu)**
- **The work is funded by U.S. Army Medical Research and Materiel Command (W81XWH-13-1-0017 PI: Dr. Bruce Spiess)**

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3. Smith CR, Parsons JT, Zhu J, Spiess BD. The effect of intravenous perfluorocarbon emulsions on whole-body oxygenation after severe decompression sickness. Diving Hyperb Med. 2012;42(1):10-7.
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Title: EVALUATION OF NONINVASIVE CARDIAC OUTPUT MONITORING IN SHEEP WITH HEMODYNAMIC INSTABILITY

Authors: Paul A. Middleton, MD, Penny S. Reynolds, PhD, Jiepei Zhu, MD PhD, Bruce D. Spiess, MD

INTRODUCTION: Noninvasive cardiac output monitoring (NICOM) uses bioreactance technology to measure the phase shift between an applied alternating current and voltage measured across the thorax; phase shifts are tightly coupled to changes in thoracic pulsatile blood flow, and hence stroke volume.[1] This technology has been validated in several patient populations, but not in the setting of acute hemorrhagic shock. In this study, we evaluated the precision and accuracy of NICOM against thermodilution-based cardiac output (T-CO) under hemodynamically unstable conditions. The objective was to compare the effect of intravascular volume depletion on measurements obtained by NICOM versus the gold standard PAC. The hypothesis was that bioreactance measured CO was able to follow very low CO when PA catheter monitoring might be unable to obtain data.

METHODS: After IACUC approval, twenty male juvenile Dorset sheep were studied for a hemorrhagic shock model of resuscitation. Animals were anesthetized with isoflurane after initial induction, intubation, and ventilation. Animals were continuously monitored with cut downs on the femoral artery (BP) and the femoral vein (CO). NICOM required placement of four electrode stickers on the thorax, forming a box around the heart. Animals were stabilized then hemorrhaged to a MAP <33 mmHg and held at that level for 1 hr prior to resuscitation. Resuscitation was IV infused hetastarch, equal volume to blood loss, as animals were stabilized at a MAP >55 mmHg. Animals were observed for 1 hr after resuscitation. The data collected included CO and MAP every 20 min from the acutely hemorrhaged sheep from baseline to 1 hr post-resuscitation. Data were compared between measures and over time.

RESULTS: MAP at baseline averaged 68 mmHg; avg baseline CO was 2.0 and 2.9 L/min for PAC and NICOM respectively. NICOM measurements were consistently higher than PAC (0.85 L/min; SE 0.16 L/min; $p<0.0001$; limits of agreement -1.6, 3.3 L/min). The greatest differences between methods occurred at the end of shock, coinciding with the greatest variability in hemodynamic stability (MAP σ=23 mmHg). The correlation between PAC and NICOM over the entire process was moderate ($r = 0.65$); although 14/20 animals showed excellent congruence between methods (cross correlation $r>0.89$ at zero lag), PAC readings for 6/20 sheep consistently lagged behind NICOM. Although NICOM readings had higher intrinsic variability (NICOM = 0.98; versus PAC = 0.13), PAC readings were less reliable with 24 dropped or out-of-range observations (CO < 0.1 L/min) during shock, versus none with NICOM.

CONCLUSION: NICOM outperformed PAC when measuring CO in hemodynamically unstable subjects with rapidly changing MAP. NICOM responses were consistently reliable with acceptable accuracy in a clinically realistic shock model. Availability of such a tool will allow clinicians to have information about CO in patient when the T-CO method is not feasible to help diagnose and guide therapy.

REFERENCES:

1. *Multicenter Evaluation of Noninvasive Cardiac Output Measurement by Bioreactance Technique*. Journal of Clinical Monitoring and Computing 2008; 22: 113-119.

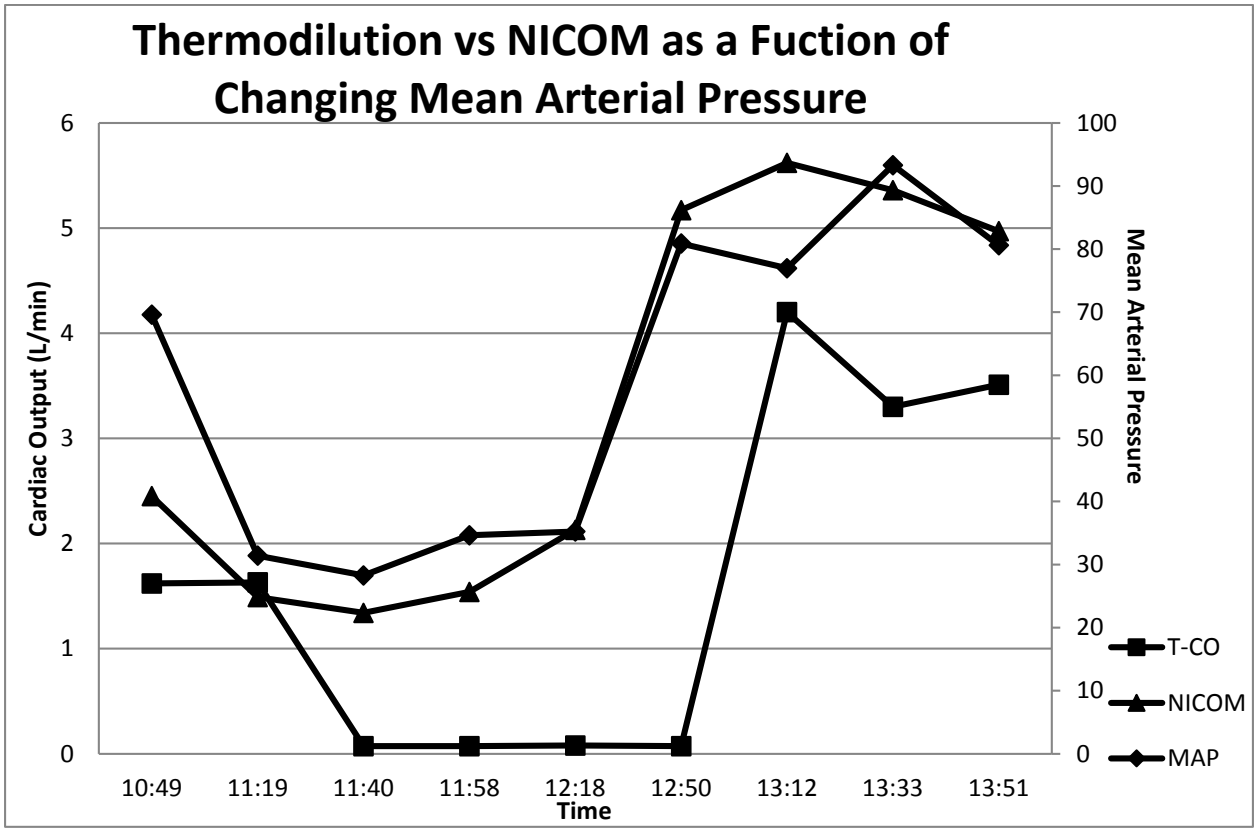
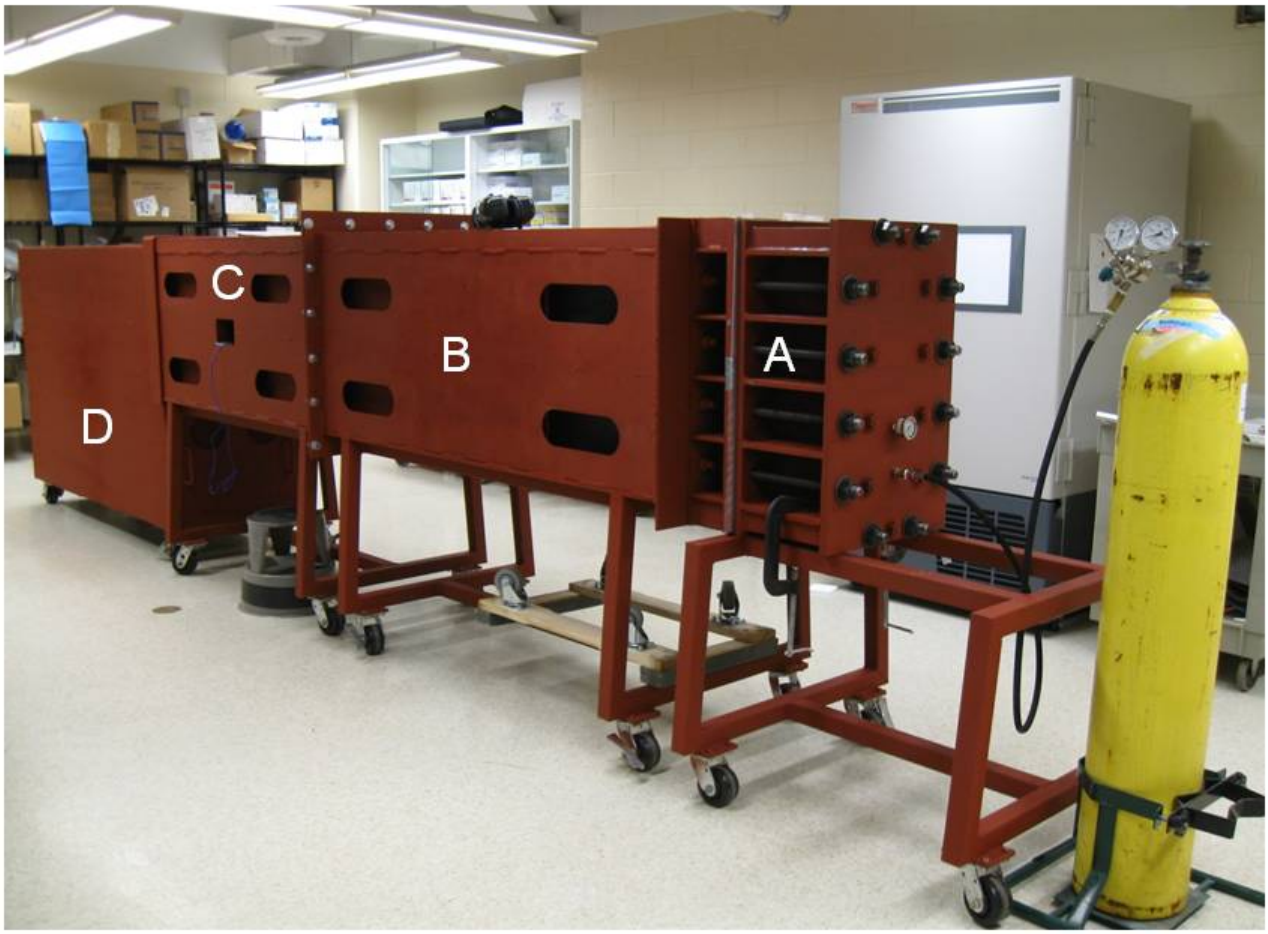


Figure I: MAP and corresponding CO over a period of hemorrhage and resuscitation, demonstrating the unreliable nature of thermodilution at low intravascular volume.

Advanced Blast Simulator Characterization for Blast TBI / Hemorrhage Polytrauma Model Development:

Simulation of simple free field Friedlander blast overpressure waves will be conducted in the laboratory using a 2.5ft diameter Advanced Blast Simulator (ABS) designed by Dave Ritzel (Dyn-Fx consulting) and Steve Parks (Operations Research Applications, or ORA, inc.), blast physicists and engineers, respectively, with over 30 years of experience in the field. The ABS was designed to accurately reproduce free field blast overpressure waves as would be experienced on the battlefield as a result of an IED detonation. The ABS (see **Figure 1** below) consists of driver (A), transition (B), test (C), and end wave eliminator (D) sections. An air blast pressure wave is created by building up pressure behind mylar membrane clamped between the driver (A) and transition section (B). Once critical pressure is reached, the mylar membrane ruptures sending a pressure wave through the transition section (B) and into the test section (C). The test section is where the backward reflective pressure wave catches up to the forward pressure wave creating the simple free field Friedlander wave. The animal is exposed to the air blast wave in the test section (C). Intensity of air blast wave is controlled by manual rupture of the mylar membrane or critical rupture pressures achieved with membranes of various thickness.

Figure 1



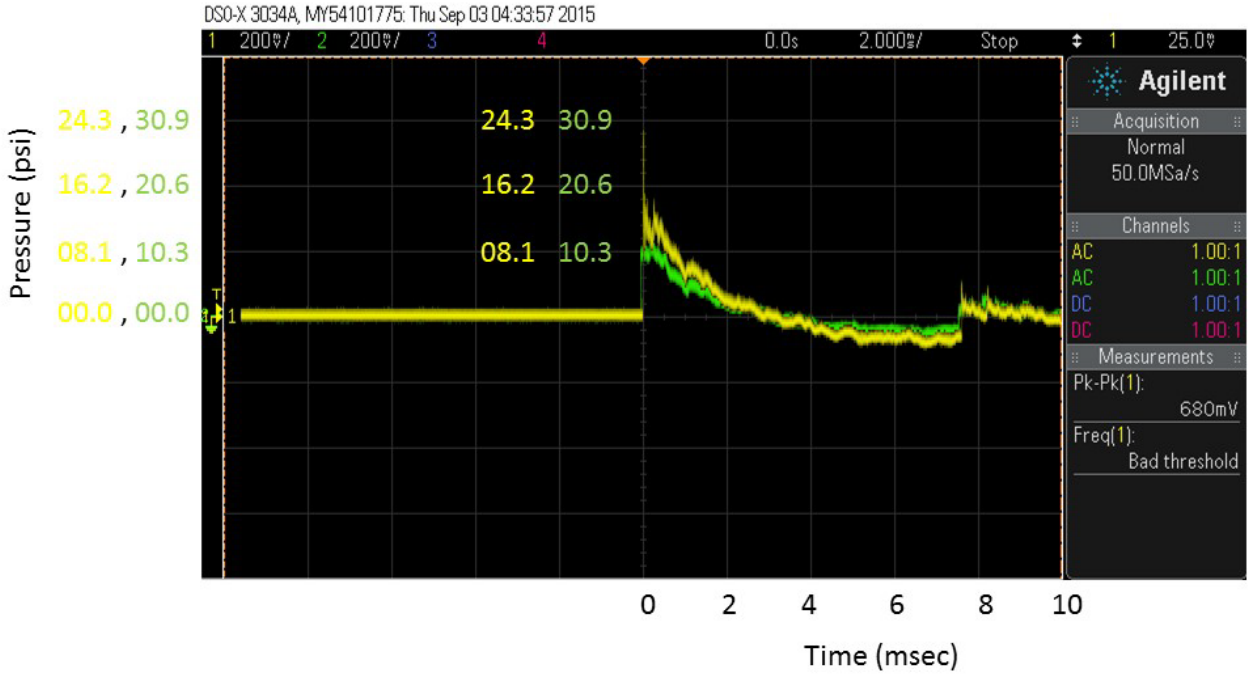
Blast wave peak pressure, pulse duration, dynamic and static pressures are assessed by a PCB 137A24 static blast pressure sensor combined with PCB 113B21 or PCB 113B22 dynamic blast pressure sensors recorded

with Agilent Technologies 3034A 350 MHz 4 GSa/s 4 channel oscilloscope. Static pressures are measured with the sensor at a 90 degree angle to the oncoming pressure pulse and dynamic pressures are measured with the sensor facing the oncoming pressure pulse.

During the current reporting period, furthur advances have been made in the characterization of the blast pressure trace. **Figure 2** shows a pressure trace obtained in the test section of the ABS using 0.015 mil mylar membrane. The yellow tracing represents the dynamic pressure as measured by PCB 113B21 dynamic pressure transducer (24.64 mV/psi, PCB Piezotronics Inc., Virginia Beach, VA). The green tracing represents the static pressure as measured by PCB 137A24 static pressure transducer (19.48 mV/psi, PCB Peizotronics Inc., Virginia Beach, VA). Both waveforms captured simultaneously using Agilent DSO-X 3034A oscilloscope (Agilent Technologies, Santa Clara, CA).

Once the mylar membrane ruptures, both a pressure wave and jet stream are sent through the ABS. The dynamic transducer will measure both the jet stream and the pressure wave while the static transducer will measure only the pressure wave. It can be seen that in the test section, the jet stream has mostly dissipated and the majority of the tracing measured by the dynamic transducer is the result of the desired pressure wave.

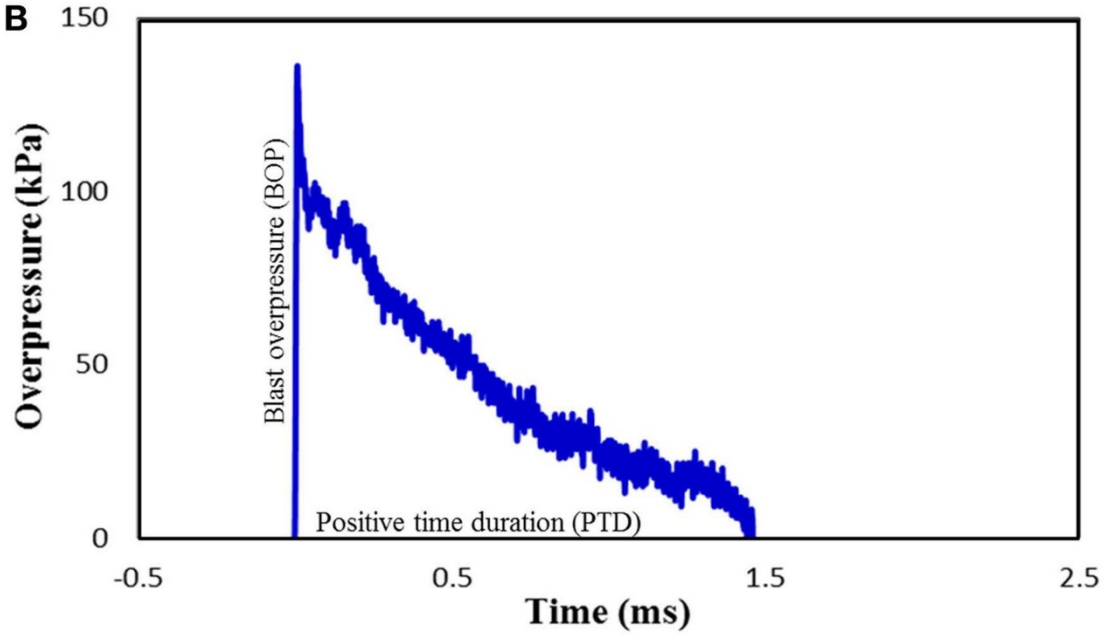
Figure 2



Furthur evidence that the wave front experienced by the impacted subject in the test section of the ABS closely mimics what would be experienced in an open field by exposure to IED can be seen by comparison of the trace in **Figure 2** to that of the trace in **Figure 3**.

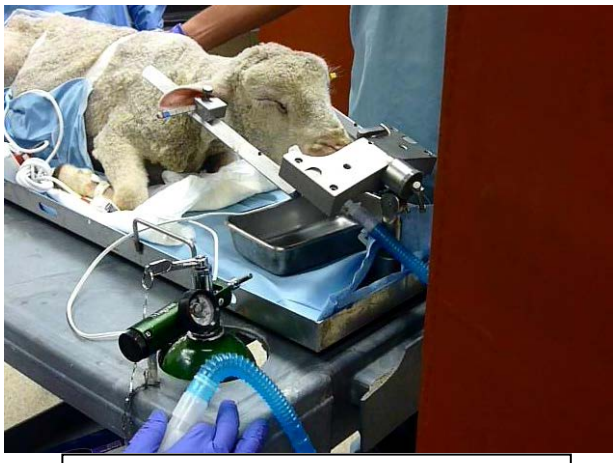
Figure 3 shows blast pressure wave of 1.8 kg of C4 measured at 2.8 meters from the epicenter using PCB 137A22 static pressure transducer. 1kpa = 0.145 psi. From Aravind Sundaramurthy and Namas Chandra. A parametric approach to shape field-relevant blast wave profiles in compressed-gas-driven shock tube. Front. Neurol., 02 December 2014 | http://dx.doi.org/10.3389/fneur.2014.00253

Figure 3

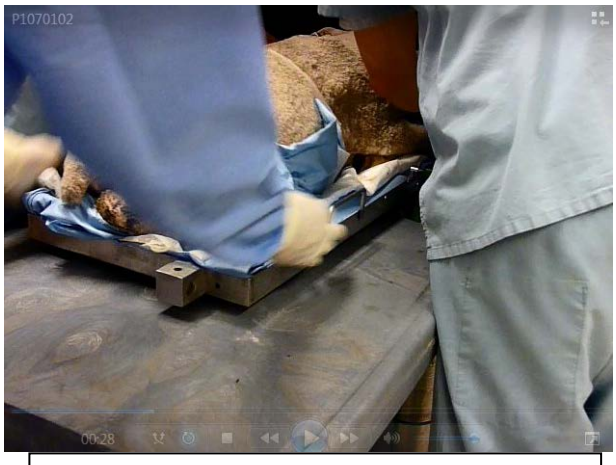


It can be seen that peak pressure, pulse duration, and pressure wave profile is very similar comparing the tracing from the ABS and the C4. Comparing peak overpressures in Figure 2 and 3, it is estimated that a subject in the test section of the ABS using the 1.5m mil mylar membrane would experience a blast pressure pulse equivalent to about 2.5lbs of C4 from a distance of approximately 10 feet from the epicenter.

Pictures for the development of polytrauma (blast injury plus hemorrhagic shock) in VCU laboratories.



Ready to move into blast chamber



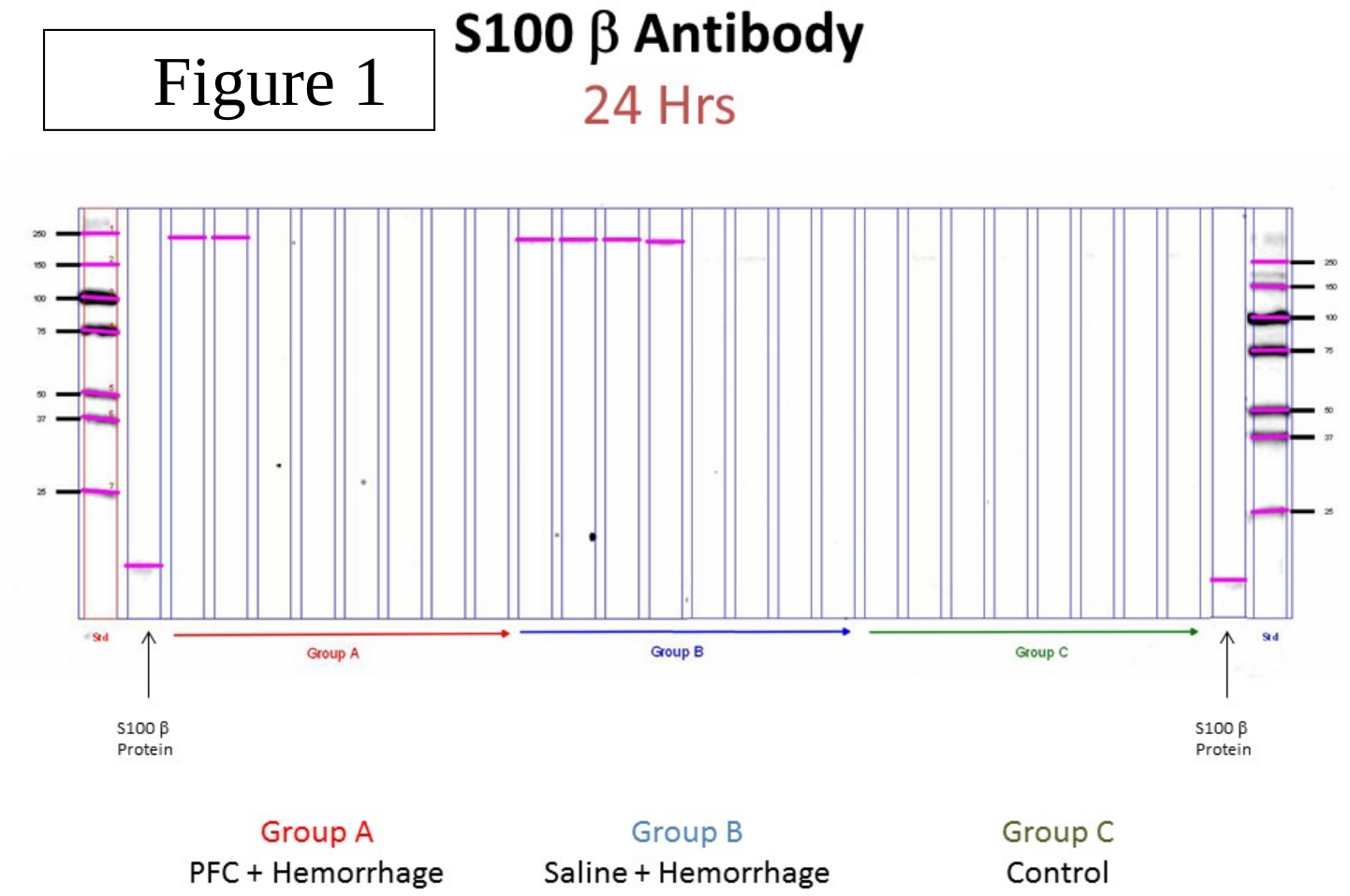
Moving into the blast chamber for blast

Biomarkers of Neuronal Damage in Hemorrhage Sheep:

S100β is a 21 kDa Ca^{2+} binding homodimer of the S100 family of multifunctional proteins with regulatory roles in a variety of cellular processes. S100β is the most studied of the S100 family and it is often utilized as a marker of global brain injury (due to its abundance in astrocytes and cellular release from astrocytic activation) and an indicator of blood-brain barrier integrity (often found in csf and blood following multiple brain pathologies). S100β can be detected in serum/plasma and CSF samples and is negatively correlated with outcome associated. S100β has been detected in brain tissue, cerebrospinal fluid, and plasma, and thus can serve as a minimally invasive method of analyzing potential brain damage and efficacy of PFC treatment.

Male sheep (20-30kg) were anesthetized, intubated, and ventilated on room air with 1-2% isoflurane. Animals were instrumented for measurement of vitals, hemodynamics, and sampling of blood for gases, biochemistry, and hematologic evaluation. Arterial blood was removed 3ml/kg/min until MAP below 40mmHg. Once MAP returned to 50 (or 15min), blood was removed 2ml/kg/min until MAP below 35mmHg. Once MAP returned to 40 (or 15min), blood was removed 1ml/kg/min until MAP below 25mmHg. Sheep remained at MAP 25-30mmHg for 1hr. Sheep were then resuscitated with minimal volume hetastarch (until MAP 60mmHg) then given either 6cc/kg saline (n=8) or Oxygent PFC (n=8). Control animals (n=8) underwent the same procedures as hemorrhagic sheep except blood was not withdrawn nor fluid resuscitation administered. Plasma samples were collected 24 hours prior to hemorrhage (baseline) and at 24 hours, 4 days, and 7 days post hemorrhage. Plasma sample proteins were balanced by UV-Visible spectrophotometry, resolved by SDS-PAGE, and transferred to nitrocellulose. The resulting western blots were probed for S100β using a commercially available

Figure 1



antibody, visualized by chemiluminescence, and densitometerically analyzed. Figure 1 shows western blot of plasma samples from control, hemorrhage sheep treated with PFC, and hemorrhage sheep treated with saline at

24 hours following hemorrhage. Analysis of the blot at 10.5 kDa at all time points in all samples does not show any expression of S100β. The results suggest that there is no astrocytic activation nor breakdown of the blood brain barrier 24 hours following hemorrhagic shock in the currently utilized sheep model. This data further supports the lack of detection of alpha II spectrin breakdown products at all time points in this model previously reported in the 2015-July-15 quarterly report.

Biomarkers of Neuronal Damage in Hemorrhage Sheep:

Alpha-II spectrin is a 280 kDa cytoskeletal protein found in neurons whose breakdown products are established biomarkers of neuronal damage. Connected with necrosis or apoptosis, calpain or caspase-3 cleave alpha-II spectrin into different sized segments. Necrotic induced calpain activity is associated with 150 and 145 kDa breakdown products. Apoptosis induced caspase-3 activity is associated with 150 and 120 kDa breakdown products. Alpha-II spectrin breakdown products have been detected in brain tissue, cerebrospinal fluid, and plasma, and thus can serve as a minimally invasive method of analyzing potential brain damage and efficacy of PFC treatment.

Male sheep (20-30kg) were anesthetized, intubated, and ventilated on room air with 1-2% isoflurane. Animals were instrumented for measurement of vitals, hemodynamics, and sampling of blood for gases, biochemistry, and hematologic evaluation. Arterial blood was removed 3ml/kg/min until MAP below 40mmHg. Once MAP returned to 50 (or 15min), blood was removed 2ml/kg/min until MAP below 35mmHg. Once MAP returned to 40 (or 15min), blood was removed 1ml/kg/min until MAP below 25mmHg. Sheep remained at MAP 25-30mmHg for 1hr. Sheep were then resuscitated with minimal volume hetastarch (until MAP 60mmHg) then given either 6cc/kg saline (n=8) or Oxygent PFC (n=8). Control animals (n=8) underwent the same procedures as hemorrhagic sheep except blood was not withdrawn nor fluid resuscitation administered. Plasma samples were collected 24 hours prior to hemorrhage (baseline) and at 24 hours, 4 days, and 7 days post hemorrhage. Plasma samples proteins were balanced by UV-Visible spectrophotometry, resolved by SDS-PAGE, and transferred to nitrocellulose. The resulting western blots were probed for Alpha-II spectrin breakdown products using a commercially available antibody, visualized by chemiluminescence, and densitometerically analyzed. Figures 1-4 show western blots of plasma samples from control, hemorrhage sheep treated with PFC, and hemorrhage sheep treated with saline at baseline, 24 hours, 4 days, and 7 days following hemorrhage. Analysis of the blots between 100 and 150 kDa at all time points in all samples does not show any expression of Alpha-II spectrin breakdown products.

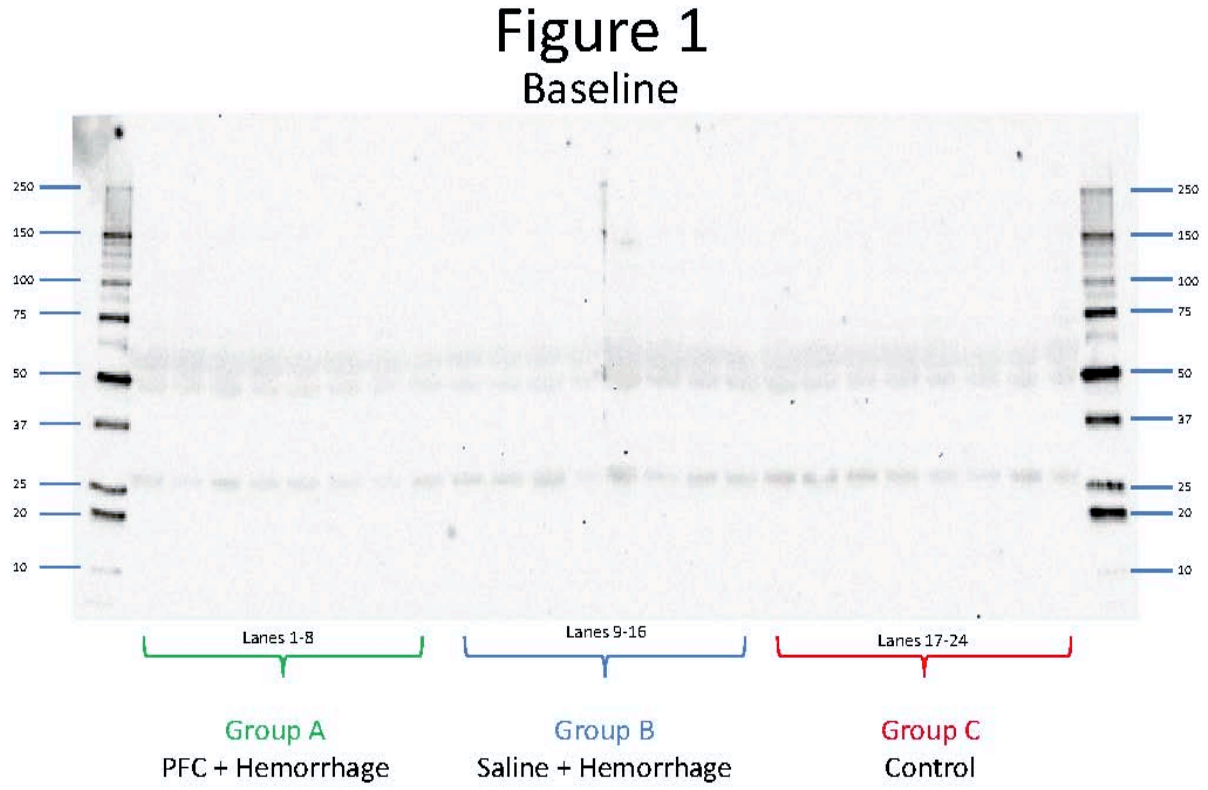
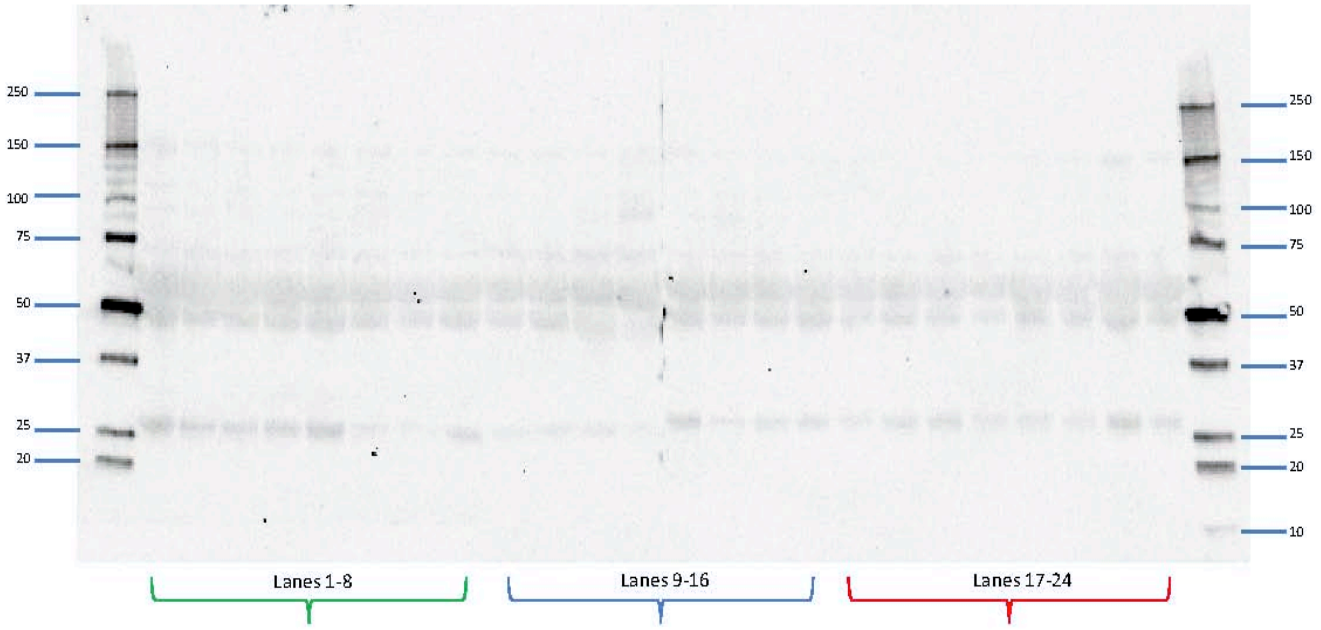


Figure 2

24 Hours



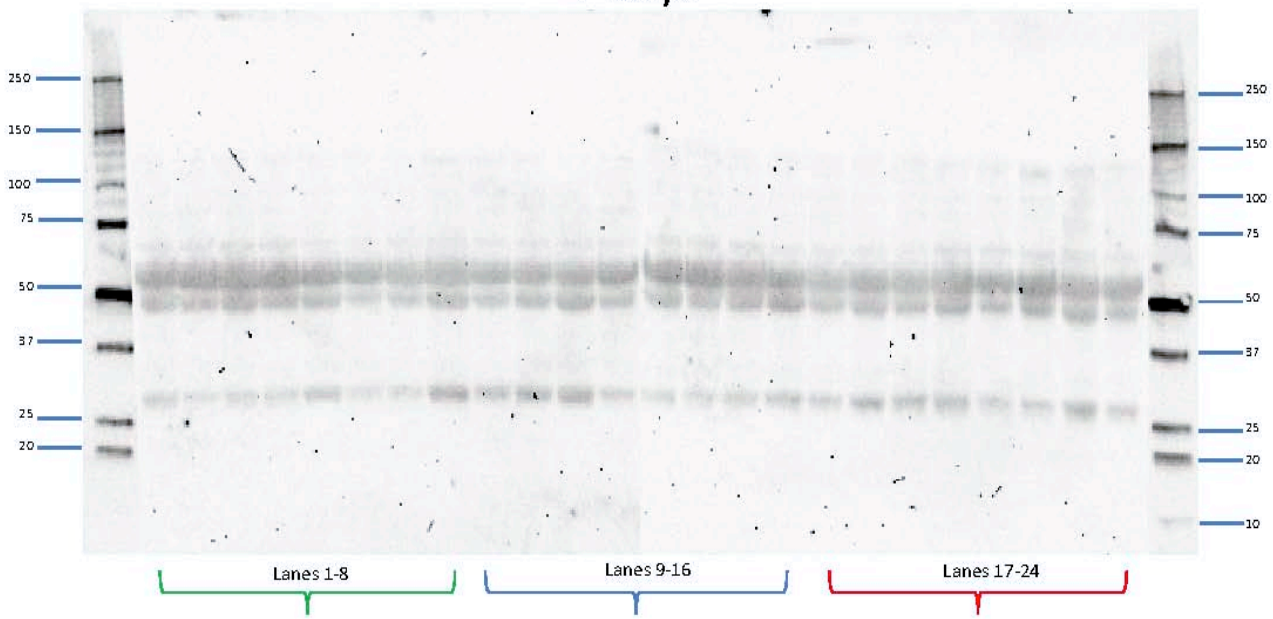
Group A
PFC + Hemorrhage

Group B
Saline + Hemorrhage

Group C
Control

Figure 3

4 Days



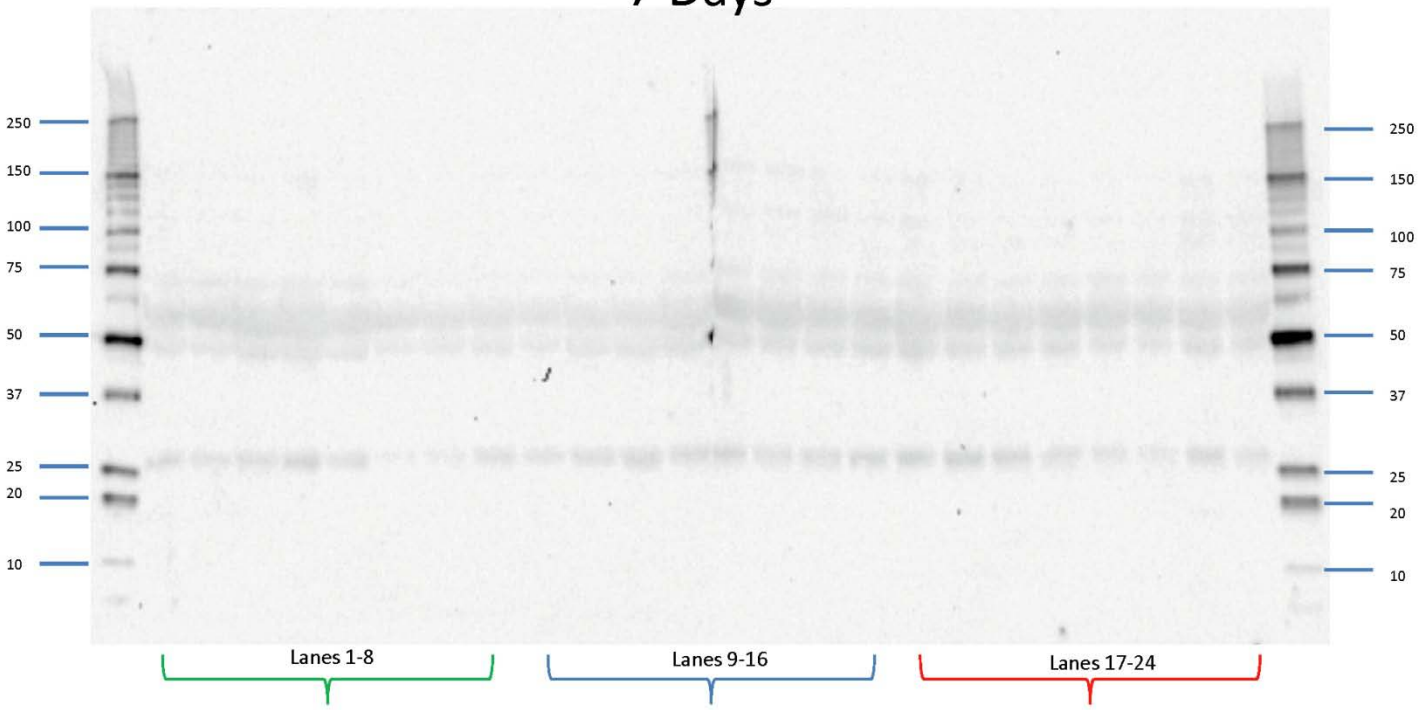
Group A
PFC + Hemorrhage

Group B
Saline + Hemorrhage

Group C
Control

Figure 4

7 Days



Group A
PFC + Hemorrhage

Group B
Saline + Hemorrhage

Group C
Control

Parameter	Baseline		1 Hour		24 Hour		96 hour		7 Days	
Hemorrhage	**Mean**	*SE*	**Mean**	*SE*	**Mean**	*SE*	**Mean**	*SE*	**Mean**	*SE*
NATEM CT (sec)	546.33	94.66	471.78	38.54	670.00	96.53	682.11	102.46	663.33	103.93
	479.60	43.20	562.30	46.78	537.20	50.12	400.90	35.21	422.40	37.07
	550.70	48.64	539.30	30.73	566.60	73.67	552.10	34.37	508.80	52.48
	487.50	45.80	562.30	41.58	537.20	58.42	400.90	59.32	422.40	48.36
NATEM CFT (sec)	170.56	28.14	169.22	11.64	186.56	19.40	195.67	33.66	178.22	45.08
	144.90	21.72	193.20	19.27	153.30	10.64	113.30	11.52	104.10	10.20
	168.90	20.43	157.70	17.19	166.80	25.17	154.80	15.94	141.10	15.05
	142.70	16.86	193.20	13.66	153.30	19.05	113.30	16.53	104.10	16.80
NATEM MCF (mm)	72.44	1.71	64.67	1.88	76.33	1.33	79.56	1.23	81.11	1.73
	75.40	1.78	65.20	1.75	74.10	1.89	78.30	2.24	76.40	1.43
	73.10	1.89	73.60	2.25	76.30	1.89	76.70	1.32	76.90	1.67
	73.90	1.43	65.20	1.19	74.10	1.26	78.30	1.04	76.40	1.92
NATEM Angle (%)	61.67	3.48	58.78	1.74	59.00	2.57	61.00	3.82	64.56	4.46
	65.20	2.89	56.60	2.66	63.10	1.67	70.30	2.01	70.90	1.72
	61.70	3.03	62.80	2.30	62.20	2.96	63.90	2.38	65.80	2.11
	64.40	2.64	56.60	1.99	63.10	2.76	70.30	2.34	70.90	2.63
INTEM CT(sec)	159.78	12.61	187.33	14.39	177.78	12.08	156.89	13.25	171.89	13.94
	144.60	7.07	193.70	6.56	151.40	5.74	139.20	5.94	155.90	5.16
	157.60	8.55	156.10	8.83	163.30	7.78	159.80	4.23	149.30	9.81
	156.10	7.95	193.70	8.60	151.40	11.34	139.20	6.82	155.90	9.44
INTEM CFT (sec)	72.00	4.57	155.89	16.29	78.44	10.40	48.67	3.13	44.89	2.82
	56.40	4.53	148.60	18.47	63.20	7.18	49.50	6.86	45.60	3.58
	67.30	8.57	67.80	22.36	51.40	9.03	54.00	2.84	48.90	2.00
	53.10	3.08	148.60	7.10	63.20	3.00	49.50	3.68	45.60	3.01
INTEM MCF (mm)	75.89	1.22	136.67	70.06	152.44	74.95	81.67	0.93	83.44	1.36
	78.70	1.28	67.00	1.91	76.90	1.58	78.50	2.00	79.10	1.59
	77.20	1.55	76.50	2.33	79.90	1.41	80.20	1.28	80.20	1.24
	78.40	0.88	67.00	1.35	76.90	1.02	78.50	1.31	79.10	1.55
INTEM Angle (%)	76.44	0.82	61.22	2.46	77.33	1.04	80.22	0.55	80.89	0.39
	78.60	0.83	62.80	2.84	78.50	0.86	80.70	0.76	81.30	0.56
	76.90	1.45	76.40	2.85	79.60	0.95	79.30	0.61	79.90	0.45
	79.20	0.57	62.80	1.44	78.50	0.58	80.70	0.62	81.30	0.64
EXTEM CT (sec)	74.44	5.83	94.00	10.87	68.78	5.58	78.22	9.71	78.11	8.33
	80.30	3.05	124.20	5.25	76.90	2.69	79.40	3.91	81.30	2.74
	72.30	6.06	66.10	4.80	71.40	5.46	83.30	9.06	79.80	8.28

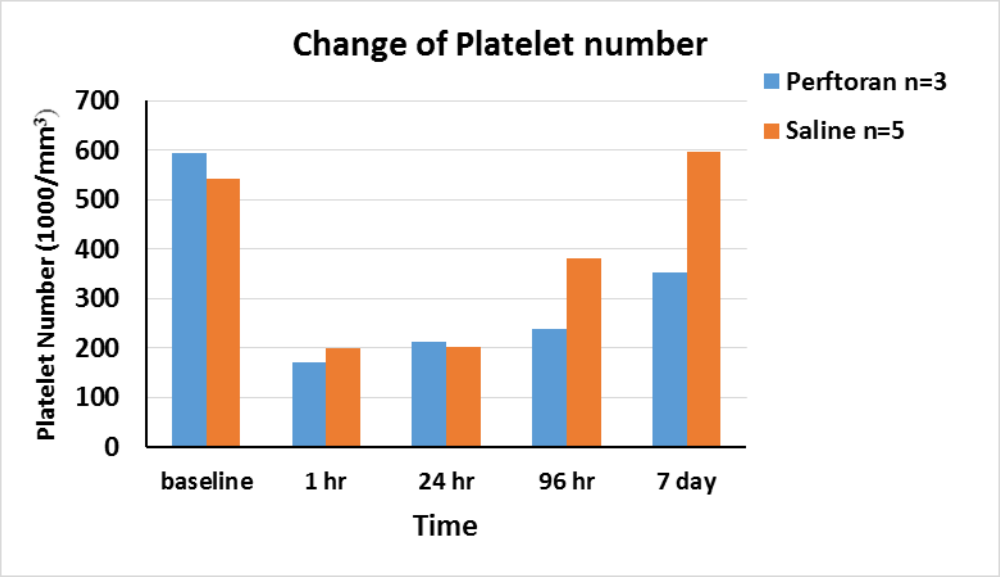
	62.80	6.22	124.20	6.99	76.90	7.09	79.40	10.71	81.30	9.57
EXTEM CFT (sec)	96.11	5.87	224.00	17.88	104.22	18.48	52.56	5.08	57.44	5.01
	76.80	5.49	221.90	20.92	71.40	8.26	57.60	7.17	64.00	4.01
	88.20	9.22	104.60	20.63	67.80	9.73	71.50	5.78	73.20	21.46
	84.90	6.69	221.90	6.40	71.40	5.20	57.60	4.69	64.00	5.91
EXTEM MCF (sec)	75.89	1.21	66.78	2.01	76.33	1.27	82.11	0.95	83.22	1.52
	78.60	1.55	69.20	1.96	77.50	1.61	78.90	2.21	79.50	1.61
	77.30	1.58	76.40	2.03	79.80	2.75	80.10	1.34	80.50	1.39
	78.10	0.84	69.20	1.17	77.50	0.87	78.90	1.39	79.50	1.81
EXTEM Angle	74.44	1.72	54.56	3.19	77.89	2.08	81.44	0.50	160.33	81.46
	78.20	1.22	55.90	3.37	79.70	0.88	80.90	0.69	79.30	0.86
	75.60	1.77	74.50	3.69	79.40	0.80	78.50	0.90	77.40	0.87
	75.90	1.28	55.90	1.29	79.70	0.76	80.90	0.83	79.30	1.28
Platelet Count	533.89	67.55	271.67	61.50	271.67	54.11	340.78	51.09	460.44	26.69
	447.47	38.76	153.48	20.87	188.47	18.14	355.45	62.11	447.27	30.99
	498.70	38.56	336.40	41.87	484.90	35.46	497.10	44.30	528.50	78.82
	562.00	60.10	153.48	48.70	188.47	56.65	355.45	49.42	447.27	39.94
Fibrinogen	172.22	10.28	109.22	7.95	225.78	17.20	311.00	29.86	260.56	30.12
	226.20	21.29	127.00	11.87	220.30	25.64	237.50	27.61	192.80	13.41
	191.70	19.25	170.20	15.19	214.10	16.56	259.30	39.38	237.40	44.63
	176.80	9.19	127.00	13.66	220.30	17.94	237.50	43.95	192.80	47.59
Coll/Epi PFA	247.22	21.54	300.00	0.00	270.44	15.27	223.22	24.92	222.11	20.98
	217.10	24.94	291.00	9.00	239.40	23.46	184.40	24.34	225.00	26.58
	218.30	25.08	265.80	13.20	198.10	18.15	213.80	16.45	144.60	21.12
	227.50	26.87	291.00	18.03	239.40	30.14	184.40	25.86	225.00	9.12
Coll/ADP PFA	84.44	7.00	124.33	26.03	193.22	34.60	85.56	12.53	85.44	6.81
	106.50	21.97	171.60	28.21	114.50	14.17	127.00	26.08	78.60	7.76
	143.60	27.25	71.10	28.12	82.80	22.01	107.70	15.30	75.90	6.13
	74.40	6.37	171.60	5.24	114.50	9.14	127.00	25.37	78.60	8.28
FOT (min)	9.22	2.20	8.33	1.00	11.22	1.81	11.67	2.33	10.33	1.94
	7.90	0.99	10.70	1.59	8.40	0.83	5.90	0.71	6.00	0.68
	8.90	0.90	8.60	0.96	8.10	1.54	8.80	0.91	7.30	1.34
	7.80	1.02	10.70	0.97	8.40	1.11	5.90	1.24	6.00	0.98
CEM (kdynes/cm2)	37.95	8.80	17.06	2.69	25.21	7.57	31.60	9.11	39.38	8.84
	51.46	7.55	2.55	0.62	47.72	6.40	70.38	7.97	57.01	7.62
	33.11	5.07	38.12	4.78	48.63	8.63	39.36	5.77	54.88	11.28
	38.69	3.38	2.55	5.46	47.72	6.30	70.38	5.99	57.01	6.45

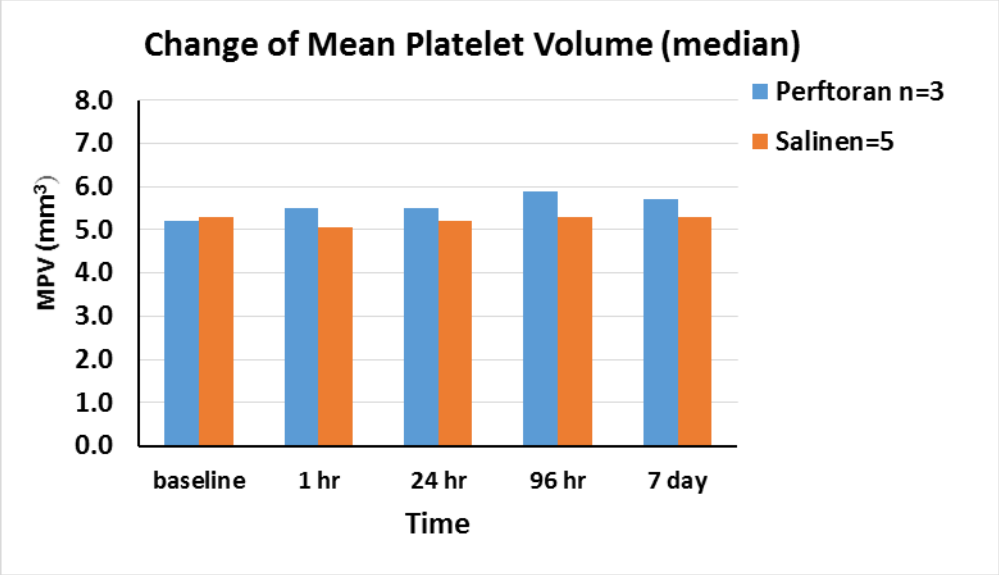
PCF (kdynes)	8.51	2.20	4.54	1.06	5.82	1.77	7.21	2.19	11.09	2.92
	9.66	2.08	1.66	0.30	8.42	1.50	13.78	2.21	12.32	1.83
	6.46	1.43	8.06	1.05	10.93	2.50	8.31	1.93	12.51	3.44
	9.64	1.76	1.66	1.55	8.42	1.99	13.78	1.66	12.32	2.22
Liver Enzyme	11.11	1.64	10.22	1.40	33.78	7.54	31.33	7.21	19.00	3.76
	18.70	1.33	12.89	1.07	29.22	4.10	28.20	3.33	20.80	2.42
	17.40	1.08	15.50	1.29	33.60	4.96	40.70	3.54	25.33	3.36
	18.50	1.46	12.89	0.73	29.22	3.53	28.20	5.66	20.80	2.67
Platelets CD62p										
vWF: Ag										
Collagen Agg (Ohms)	8.72	0.53	4.39	1.02	5.56	0.78	8.33	0.46	10.39	0.90
	7.95	0.59	3.78	0.83	6.06	1.13	8.50	1.00	10.50	0.89
	9.22	0.70	9.43	0.82	9.56	0.82	8.50	0.77	10.31	0.78
	9.39	0.56	3.78	0.69	6.06	1.04	8.50	0.84	10.50	0.86
ADP Agg (Ohms)	7.94	0.71	6.00	0.75	7.00	1.30	9.61	0.74	9.50	0.60
	9.30	1.02	6.85	0.67	9.00	0.96	10.35	1.04	11.64	1.04
	10.28	0.77	9.39	0.63	10.17	0.75	8.89	0.71	11.00	0.86
	8.83	1.33	6.85	0.64	9.00	1.26	10.35	0.36	11.64	0.89
CAT lag	4.78	0.84	5.32	0.40	6.18	0.26	5.80	0.83	6.61	0.26
	7.38	0.68	8.51	0.76	6.83	0.25	7.95	0.73	7.45	0.61
	6.22	0.46	5.22	0.26	6.80	0.47	6.62	1.25	6.62	0.79
	4.99	0.19	8.51	0.40	6.83	0.85	7.95	0.40	7.45	0.98
CAT ETP	946.63	265.29	767.97	106.86	806.00	147.99	1496.88	228.75	1261.41	206.01
	1066.86	126.38	732.11	137.55	1032.55	128.97	1404.96	152.87	1357.70	159.44
	1283.80	211.84	1161.50	139.60	1220.92	168.03	1338.69	120.54	1453.35	185.26
	1141.66	106.75	732.11	100.08	1032.55	95.53	1404.96	44.68	1357.70	139.30
CAT Peak	48.09	17.04	42.71	5.00	40.95	8.12	85.86	17.90	75.13	14.79
	61.06	9.33	38.16	6.65	52.25	6.71	88.98	12.19	97.28	14.64
	66.07	14.36	65.32	61.54	62.23	7.40	79.69	10.82	86.95	15.28
	74.67	9.31	38.16	9.75	52.25	6.63	88.98	7.88	97.28	10.97
CAT Tip	16.24	1.47	15.09	1.81	16.72	0.94	18.53	1.84	19.09	1.39
	19.49	1.11	20.93	1.34	19.80	0.78	17.85	1.03	16.54	0.72

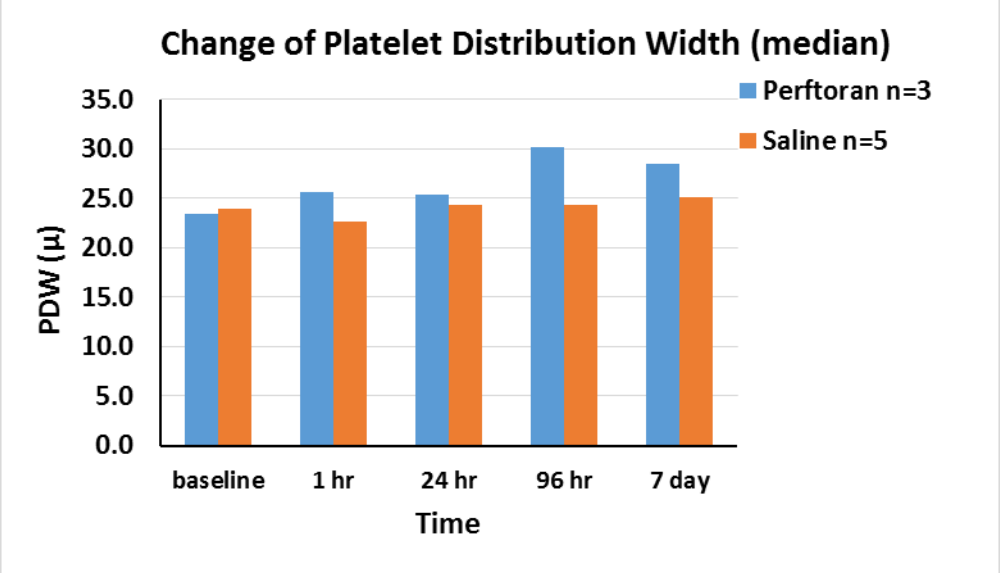
	18.97	1.02	16.86	0.84	19.40	1.22	18.58	1.38	17.66	1.95
	15.62	0.92	20.93	0.92	19.80	0.81	17.85	1.14	16.54	1.15
Neutrophils (HM5)	2.00	0.19	0.84	0.21	2.09	0.28	2.21	0.37	1.73	0.21
	2.59	0.48	1.66	0.28	2.90	0.38	2.59	0.31	2.30	0.26
	2.34	0.41	2.29	0.24	4.03	0.66	2.01	0.59	2.96	0.26
	2.99	0.45	1.66	0.32	2.90	0.64	2.59	0.31	2.30	0.36
Monocytes (HM5)	0.05	0.00	0.02	0.00	0.05	0.00	0.05	0.01	0.04	0.00
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	0.05	0.01	0.03	0.00	0.05	0.01	0.04	0.49	0.05	0.01
	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Natem TPI	62.06	13.82	36.51	5.67	61.22	10.04	82.75	16.34	115.85	23.85
	87.84	19.07	35.61	6.89	63.94	8.32	119.00	18.68	109.68	16.95
	64.26	14.73	59.03	6.04	72.52	14.29	73.53	23.14	93.91	26.53
	73.43	11.93	35.61	6.43	63.94	13.46	119.00	9.29	109.68	17.36
Intem TPI	141.55	15.77	45.89	7.39	153.08	21.42	295.43	31.52	365.77	36.14
	224.20	30.21	51.97	9.22	209.47	50.02	279.61	39.44	290.87	41.86
	195.86	44.28	170.87	22.82	252.12	32.95	256.10	56.89	296.59	43.42
	220.43	23.22	51.97	24.89	209.47	28.87	279.61	37.22	290.87	55.00
Extem TPI	105.01	10.80	31.19	5.23	120.65	20.72	300.77	42.76	309.24	47.21
	168.26	26.52	36.76	6.31	196.58	48.28	260.94	49.88	209.13	30.71
	160.95	49.45	100.98	14.86	192.90	40.13	192.04	51.57	233.02	59.27
	138.16	15.35	36.76	11.29	196.58	23.92	260.94	26.03	209.13	62.15

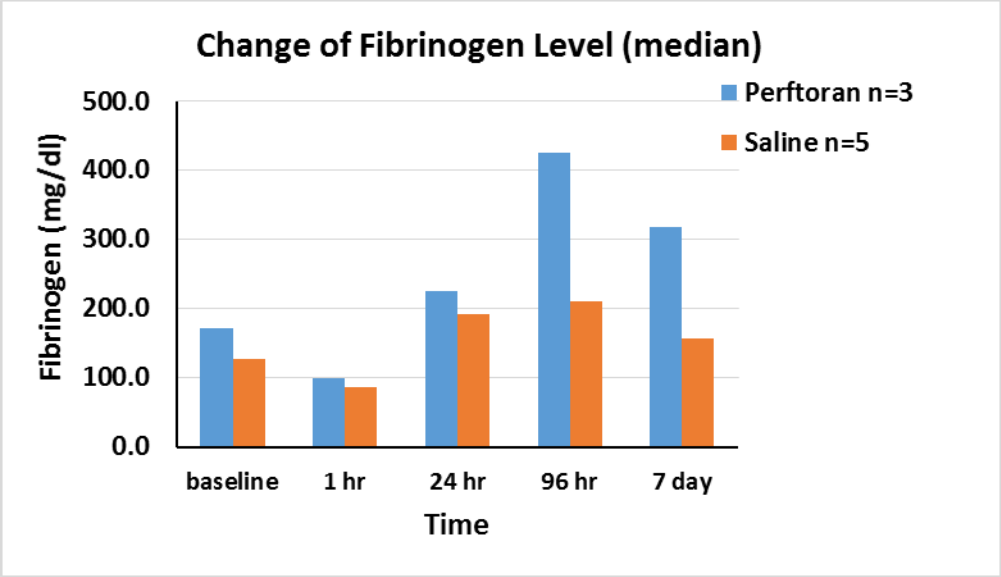
	Not enough data
	Oxygent
	Perftoran
	Saline
	Control

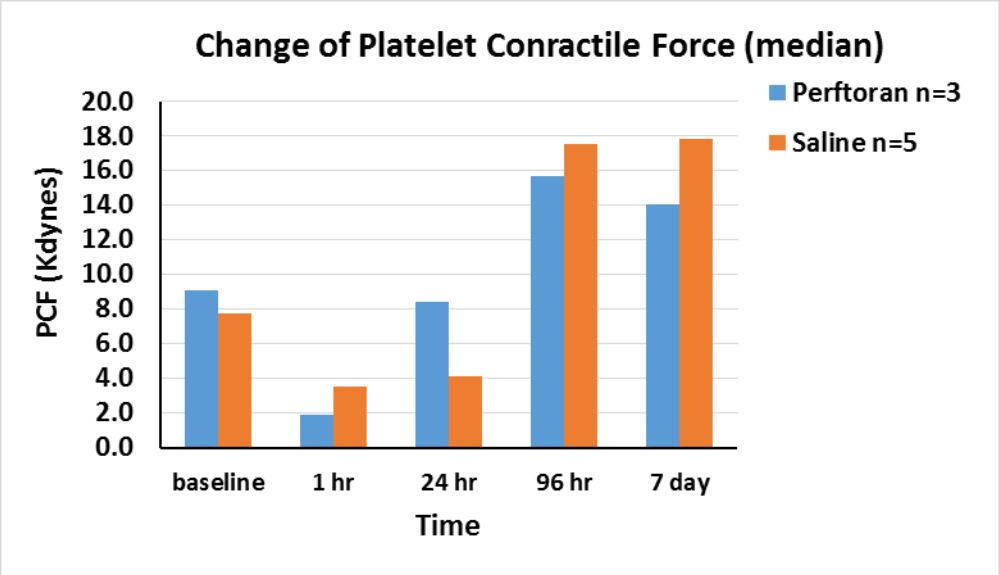
5. **Initial summary Date sheet - PFC infusion as a part of resuscitation fluid in Polytrauma sheep combined blast trauma injury following hemorrhagic shock**

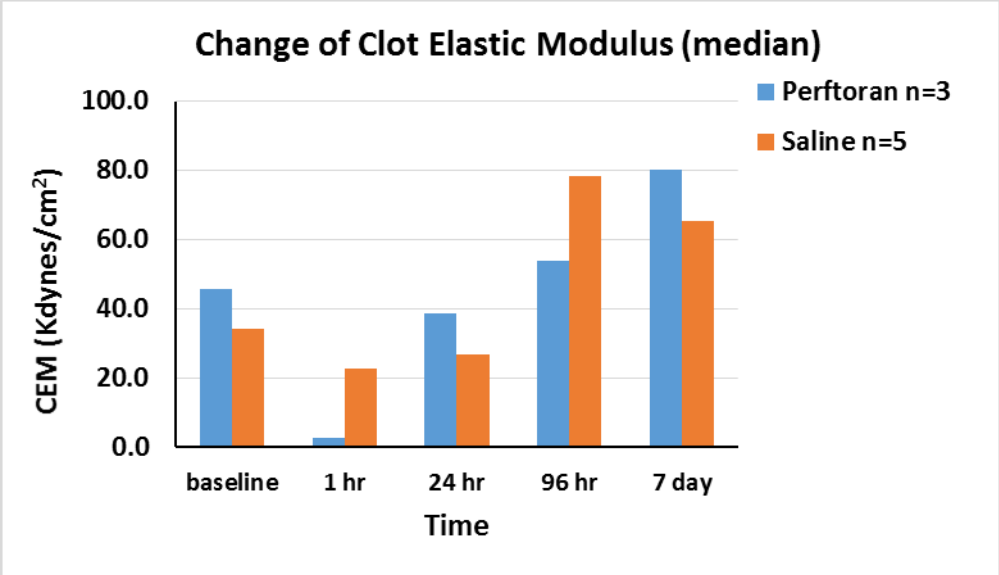


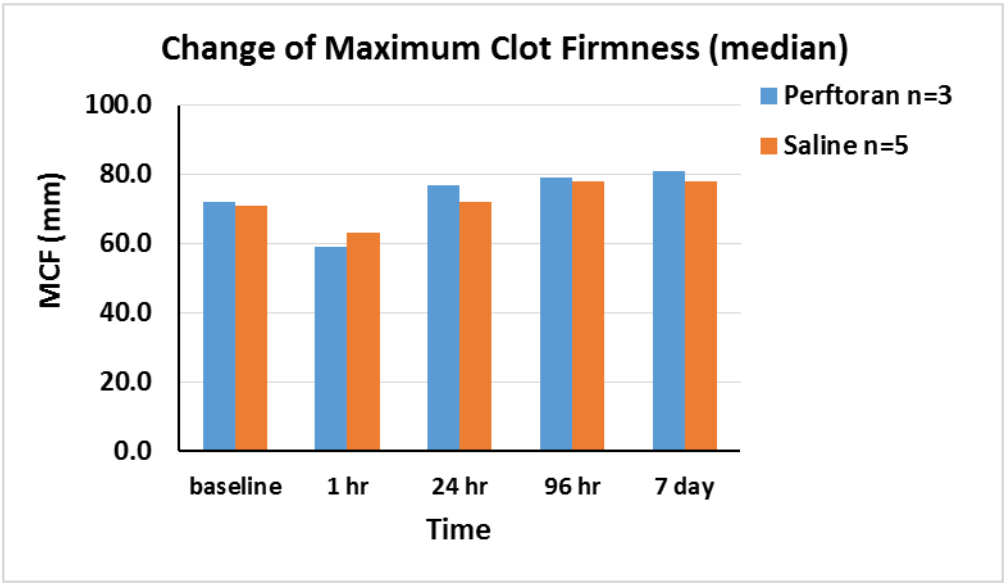





Change of Fibrinogen Level (median)
Perftoran n=3
Saline n=5
Fibrinogen (mg/dl)
500.0
400.0
300.0
200.0
100.0
0.0
baseline
1 hr
24 hr
96 hr
7 day
Time


Change of Platelet Conractile Force (median)
Perftoran n=3
Saline n=5
PCF (Kdynes)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
baseline
1 hr
24 hr
96 hr
7 day
Time


Change of Clot Elastic Modulus (median)
Perftoran n=3
Saline n=5
CEM (Kdynes/cm2)
100.0
80.0
60.0
40.0
20.0
0.0
baseline
1 hr
24 hr
96 hr
7 day
Time


Change of Maximum Clot Firmness (median)
Perftoran n=3
Saline n=5
MCF (mm)
100.0
80.0
60.0
40.0
20.0
0.0
baseline
1 hr
24 hr
96 hr
7 day
Time