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TITLE: Studies of the Effects of Perfluorocarbon Emulsions on Platelet Number and Function in Models of Critical Battlefield Injury

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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. In phase one of the study, the results showed that the sheep’s platelet number and activation were not significantly changed after PFC infusion. In 2014, PFC intravenous infusion as a part of resuscitation fluid was used in hemorrhagic sheep (PFC: oxygent, n=6; saline: n=7; surgical control: n=6). The initial results showed that the sheep’s platelet count and fibrinogen level were not significantly reduced after PFC infusion compared with non-PFC controls for the 7 survival days. Platelet contractile force (PCF, Platelet activator) also showed no significant reduction compared with control groups (saline & surgical control). Platelet morphological observation corresponds with function assays. There are no significant percentage changes in neutrophils and monocytes after PFC infusion. Therefore, intravenous infusion of Oxygen (PFC) in hemorrhagic shock sheep did not cause massive or severe coagulopathy.
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INTRODUCTION

Perfluorocarbon emulsions (PFCs) are small volume robust (temperature stable, long storage life, portable) intravenous (i.v.) fluids, easily carried by medics/corpsmen to site of first contact. PFCs enhance O2 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 O2 delivery at normal FiO2 and that perhaps the most important aspect of PFC infusion was an enhanced O2 delivery from native erythrocytes to tissues. Furthermore, it appears that PFCs enhance O2 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-O2 and Perftoran contain perfluorodecalin (88% or 20%), 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. Oxygen, another resuscitation product, contains perflubron emulsion (60%, w/v) and has an O2 carrying capacity similar to PHER-O2. In the present study, the specific aims are to answer the following: #1 Whether PFC infusion activates Plts in vivo, #2 Whether Plt/white cell clumps (micro-aggregates) 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) Using normal sheep (20-30 kg) model to test the effect of PFC intravenous infusion on platelet number and activation. 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) and 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:** Using sheep (20-30 kg) hemorrhagic shock model to test the effect of PFC intravenous infusion on platelet number and activation. Animals were anesthetized, instrumented and had bleeding 35~50% of total blood volume and maintain mean arterial pressure at 30 mmHg (±3 mmHg) for 60 minutes then resuscitated with hetastarch plus PFC (oxygent), n=6 or hetastarch plus saline, n=7 and 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) and 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. (See attached procedures)

**Year three:** Using the ovine polytrauma model of combined hemorrhagic shock and blast TBI to test the effect of PFC intravenous infusion on platelet number and activation. Volume resuscitation will occur with either hetastarch or 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 done 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 2014, total 37 animal were ordered and 44 sheep were used (control animals were reused after recovered between two control blood collections). Following chart describes the animal usage in details in 2014.

<table>
<thead>
<tr>
<th>Quarter Period</th>
<th>Sheep ordered</th>
<th>Top-load model</th>
<th>Hemorrhagic shock</th>
<th>Sum of Used</th>
<th>Model development /death</th>
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<tbody>
<tr>
<td>I</td>
<td>8</td>
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<td>10</td>
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</tbody>
</table>

**Note:**
1. Top-load study control animals were reused for surgical control in hemorrhagic shock model after 7 day survival and 7 days recovery in order to reduce animal use.
2. Model development was for hemorrhagic shock model. Some of hemorrhagic shock injured animals had difficult time to survive for 7 days and were euthanized based on the advices of our veterinarians.
3. *In quarter III, 4 animals were infected when they were shipped to VCU and were immediately euthanized by DAR.

**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 
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 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 (for details, see top-load protocol in appendices). 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 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 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 samples and resuscitation. Both sides of femoral arteries and 
veins were cut-down at 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 sample 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 completed and instrument equipped. Three-stage Hemorrhagic 
shock model was used (see attached flow chart for details). Total average amount of bleed was 32% ~ 
50% (stage II-III hemorrhagic model) and maintained the mean arterial pressure (MAP) at 30 ±3 mmHg 
for 60 minutes then starting fluid resuscitation. All hemorrhagic animals were resuscitated with 
intravenous hespan (hetastarch) first till MAP reaching 65 mmHg and stabilizing for 10 minutes, then 
intravenous infusion with 5 ml/kg of PFC (Oxygent, 60%, 3g/kg; Perftoran, 20%, 1g/kg) over 15 minutes 
or the same amount saline. Animals were closely monitoring 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 current hemorrhagic sheep 
model is estimated in 15 ~25%.
Study endpoints:

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

2. **Hemorrhagic Physiology** evaluated by monitoring blood pressure, heart rate, ECG, central venous pressure, pulmonary arterial pressure, SvO2, cardiac output and blood gas analysis during the hemorrhagic shock and resuscitation.

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 spend 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 through 7 days after the experiment. Screen monitoring and video record materials are protected and accessed only by authorized personnel following IACUC guide lines.

Statistical analysis:

Power analysis based on sheep platelet mean number was used to estimate animal numbers per experimental group. JMP pro 11.0 statistical software was used to analyze all blood sample results. Data distribution and one-way analysis of variance (ANOVA) are used to compare means. Data are compared among groups and within the group at different time points. Significant difference between means is 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 top-load 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 (2014), healthy sheep received intravenous infusion of PFC (oxygent or perftoran). Completed was a total of 32 animals in 4 groups with 8 animals per group; (study phase I). PFC animals showed no significant reduction of platelet count nor revealed significant activation of platelets when compared with control groups or compared with its baseline. Date was presented at MHSRS in August 2014 (see attachment).

2. In the current study period (2014), survival sheep hemorrhagic shock model have been
developed and being used to test the effect of PFC infusion on the platelet number and function (study phase II). Total of 13 animals were 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 and change of its activation when compared with non-PFC group or surgical control group. Based on the power analysis, each group needs 8 to 11 cases. More animal experiments will be carried out in coming year (see attachment).

PROBLEMS AND SOLUTIONS

1. At the beginning of the year, a temporary laboratory was assigned by School of Medicine of VCU because of the flood in November 2013. It took two weeks to move and establish the interim laboratory to begin running animal experiments. In October, renovation of old laboratories was completed. It also took two weeks to move back and re-establish our labs. Solution: We carefully arranged the experimental schedules and tried to catch up the schedule as best we could. In 2014, 42 animals were used for the studies compared with 2013, only 26 animals were used (see attachment).

2. In the 2nd and 3rd quarters of the year, 8 sheep were lost due to hemorrhagic procedures (n=4) or infected sheep from supply vendor (n=4). Solution: We coordinated with DAR veterinarian and talked with vendor to supply healthy sheep. We also improved animal care post hemorrhagic procedure by providing oral dextrose for 3 days after shock. In the 4th quarter, no animals were lost due to infection or hemorrhagic procedures.

3. Platelet functional assay: There were several data measurements which drifted away from baseline without clear reason. Some of the assays are still waiting for analysis until large enough sample size is obtained. Solution: Sample values were doubled and repeatedly measured to reduce assay bias.

4. Due to loss of laboratory space from the flood, the large blast simulator device could not be reassembled until late October. The schedule for testing and developing a sheep polytrauma model which combines blast injury with hemorrhage has been delayed. Solution: Double efforts to catch up to schedule in 2015.

5. Due to loss of laboratory space from the flood, testing of biomarkers of neuronal apoptosis and necrosis (alpha II spectrin) and blood brain barrier damage (S100B) was delayed. During all procedures for 2014 reporting period, blood samples were collected and stored at -80 degrees Celcius for future analysis. Solution: All samples will be analyzed during year 3 of study once Biochemistry lab is fully functional.

KEY RESEARCH ACCOMPLISHMENTS

1. In the current study period (2014), in which 15 healthy sheep received intravenous infusion of
PFC (oxygent or perforan), has been completed (total 32 animals in 4 groups with 8 animals per group; study phase I, 2013-2014). PFC animals showed no significant reduction in platelet count nor revealed significant activation of platelets when compared with control groups or compared with its baseline. Data were presented at MHSRS in August 2014 (see attachment).

2. A sheep hemorrhagic shock survival model for phase 2 of this study in 2014 was successfully developed. 25 sheep were used for the phase II study. Both the sheep top-load (phase I) and hemorrhagic shock survival models (phase II) passed VCU veterinarian observation.

3. Initial data analyses suggested that intravenous PFC infusion in healthy sheep did not result in thrombocytopenia or coagulopathy. These data are encouraging for FDA approval of further clinical trial study of PFC in the United States.

REPORTABLE OUTCOMES

1. One first year medical student was awarded medical student summer research fellowship based on this award. The work will be presented in May, 2015 (student research honor day) at VCU.

2. Based on the study results (phase I), an abstract was submitted and accepted by MHSRS 2014 as a poster presentation in August 2014. A poster was presented on student research honor day (Medical student summer research presentation on May 1, 2014) (see attachment).

3. The large animal (sheep) survival hemorrhagic shock model has been established and passed VCU veterinarian observation.

4. Initial 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 compared with non-PFC groups.

5. Manuscripts and abstracts for 2015 MHSRS are in progress based on the results of the current study (phase II).

6. Current study budget supports 3 full time employee and two part time employees.

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 is to assess PFC’s effect on platelet number and function in sheep
polytrauma model which combined blast trauma injury and hemorrhagic shock.

REFERENCES

APPENDICES

1. Poster presentation:
   a. Poster of medical student summer research
   b. Poster of MHSRS, 2014
   c. Abstract of MHSRS, 2014

2. More Platelet count and fibrinogen data & initial behavioral outcome data after PFC top-load (intravenous infusion) in healthy sheep

3. Transmission Electron Microscopy (TEM) platelet data and quantitative criteria

4. Sheep hemorrhagic shock survival model protocol and initial data

5. Renovation of Laboratories and the large blast simulator device
Morphological Characteristics of Platelets Post Perfluorocarbon Emulsion Infusion

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Abstract

Background: Perfluorocarbon emulsions (PFC) can treat traumatic injuries in the battlefield by enhanced delivery of oxygen. However, a possible side effect of PFC may be thrombocytopenia (in 30-50%) on days 2-5 after intravascular treatment. It is necessary to investigate this phenomenon to exclude platelet inflammatory/embolic safety risks before clinical trial. Methods: A total of 24 sheep were randomly divided into 3 groups (n=8/group) with a top load intravenous infusion with either PFC (Oxygent, 60%, 3 g/kg), Hespan (6% hetastarch), or control. Venous blood was sampled before the treatment (baseline) and 24 hours, as well as 4 days after treatment. Platelet rich plasma was isolated and quantitatively observed with scanning electron microscopy (SEM). Results: Morphologically, total platelet count, semi or full-activated platelets (%) were not significantly changed within or among groups (p>0.05), which is corresponding with platelet count and other coagulatory factor measurements. Conclusion: Intravenous infusion with Oxygent in healthy sheep did not cause significant reduction in number of platelets nor change their activation morphologically. Therefore, intravenous infusion with Oxygent will not cause massive or severe coagopathy.

Introduction

PFC is a non-polar solvent with enhanced respiratory gas (O2 N2 CO2) solubility found in 1968. All O2 dissolved in PFC is available for metabolic use, which is called an O2 carrier. PFC particles are 0.1-0.2 μm and get into tissues where RBCs cannot after injury. PFC can also be an extra compartment for O2 transport and has a unique affinity in low flow states. PFC shows efficacy in models (human data) of hemorrhagic shock, traumatic brain injury (TBI), spinal cord injury, decompression sickness (DCS), interstitial emphysema in dogs, stroke, etc. 9 TBI patients were treated with PFC in MVAC with good outcome (J. Spiess/Bullock, 2006). PFC may be used in the setting of trauma and for daily care of trauma patients in the ICU to improve oxygen delivery (C. Sweeney et al.). FDA requests investigation of the phenomenon to exclude platelet inflammation/embolic safety risks. Using a healthy sheep top-loaded (PFC) model and a combined hemorrhagic shock blast traumatic brain injury model to investigate the changes of platelet number and function.

Materials and Methods

Subjects and Groups:

The study was reviewed and approved by the Animal Care and Use Committee of Virginia Commonwealth University.

Total 24 Juvenile Dorset or Dorper sheep (18-32 kg body weight) were used and randomly divided into 3 groups: PFC group (n=8); Hespan group (n=8); and Control group (n=4; and saline n = 4).

Animals were given 7 days for acclimation prior to experiment, and daily vital signs are monitored.

Top-load Procedure & Blood Sample Collection:

The use samples were handled with 7 full lamb. All animals were intubated with 7.5 F tracheal tube and ventilated (Drager Fabius GS Anesthesia Machine). Animal’s neck fur was shaved and sanitized; 20 G x 2’ needle catheter was puncture into external jugular vein. 20 G x 2’ needle catheter was puncture into external jugular vein. Animal was allowed to recover from anesthesia and carry back to DAR facility after Top-load completed. Venous blood was collected via puncture needle at baseline, 0 min, 3 & 24 hour and 4 days post-top-load.

Blood sample measurement & Data Analysis

Various samples were measured for coagulatory factors including: platelet count, Birefringence, clot formation time, APTT aggregation & CO2, etc. Various samples of baseline, 24 hour and 4 days post-top-load were processed for morphological observation using scanning electron microscope.

All data was analysis using JMP 11.0.

Results

Morphology of platelet observation with scanning electron microscopy

Figure 1: Platelets were studied in control sheep (left) and in sheep that received PFC (right). Non-activated platelets are small and smooth (arrow). Semi-activated platelets are with one or two processes (white or black arrows) and increase their size.

Figure 2: Platelet Rich Plasma (PRP) preparation:

1. 4.5 ml whole blood in tube was centrifuged 100g for 20 minutes.
2. Take supernatant into 10 ml test tube, mixed with 0.1% glutaraldehyde in 0.1 M cacodylate buffer in supernatant at 60 min.
3. Centrifuge with 1200g for 15 minutes, remove all supernatant, keep solid resin.
4. Air-dry 2g (granular platelets) or wash in phosphate buffer and re-wash at 60 min.
5. Process for SEM (in VCU imaging center).

PFC was seen to stay in circulating blood at least for 24 hours.

Materials & Methods

Coagulatory factors & SEM

Experimental timeline

Baseline sample

Venous Top-loading

Venous sample at 0, 3 & 24 hour; 4 & 7 days

Coagulatory factors & SEM

Figure 3: Percentage of active platelets and semi-active platelets. Control (n=8) group (Oxygent); Hes = Hespan group (n=8); Plt = platelet. Platelets were count and calculated how many platelets were active or semi-active (see table).

Table 1: Platelet Rich Plasma (PRP) preparation:

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<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Mean</th>
<th>Std Dev</th>
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Source DF | Sum of Mean | Std Dev | Lower 95% | Upper 95% |
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<td>Total</td>
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Conclusion

After intravenous infusion PFC, there is no morphologically significant change in platelet number and function. Qualitative and quantitative observation of platelet changes in semi-activated platelets shown a bigger variable than active ones. Current results are corresponded with the changes of coagulatory factor measurements.

Therefore, intravenous infusion with Oxygent will not cause massive or severe coagopathy.

References


Acknowledgements

VCU School of Medicine Summer Research Fellowship (Mentor: Dr. Jiepei Zhu)
Core Laboratory of Research, Department of Anesthesiology & The Microscopy Core Facility of VCU
The work is funded by U.S. Army Medical Research and Materiel Command (W81XWH-10-1-0417) Dr. Bruce Spiess
**Effect of Perfluorocarbon on Platelet Number and Function after Intravenous Infusion in Sheep**

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### Introduction
- PFC is a non-polar emulsion with enhanced respiratory gas (O₂, N₂, CO₂) solubility found in 1966.
- PFC is dissolved in PFC for use in pediatric anesthesia, which is called an O₂ carrier.
- PFC and PFC particles are 0.1–0.2 µm and get into tissues where RBCs cannot carry oxygen.
- Using a healthy sheep top-load (PFC) model and a combined hemorrhagic shock traumatic brain injury model to investigate the changes of 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 24 juvenile Dorset or Dorper sheep (18–22 kg body weight) were used and randomly divided into 3 groups:
  - PFC group (n=8); Hespan group (n=8); Control group (n=8; naïve n=4 and saline n=4).
- Animals were given 7 days for acclimation prior to experiment, and daily vital signs are monitored including temperature, heart rate and respiratory rate.

### Results

**Experimental timeline**

**Conclusion:**
- There is no significant difference when groups are compared (p>0.05).
- There was no significant difference when different time points were compared (p>0.05).

**Materials & Methods**

- The experimental protocol was reviewed and approved by the Animal Care and Use Committee of Virginia Commonwealth University.
- Total 24 juvenile Dorset or Dorper sheep (18–22 kg body weight) were used and randomly divided into 3 groups:
  - PFC group (n=8); Hespan group (n=8); Control group (n=8; naïve n=4 and saline n=4).
- Animals were given 7 days for acclimation prior to experiment, and daily vital signs are monitored including temperature, heart rate and respiratory rate.

**Top-load Procedure & Blood Sample Collection:**
- Animals were intubated with 5% isoflurane and ventilated (Drager Faktor GA Anesthesia Machine) with 35% O₂, 75% N₂.
- Animals were divided into 2 groups: 5 ml/kg of saline or PFC (Oxygent, 90%) or Hespan (6% Hetastarch) were intravenous infusion with 5 ml/kg over 15 minutes.
- Venous blood was collected via jugular vein puncture at T0 (baseline), T1 (0 min), T2 (3 hours), T3 (24 hours) and T4 (7 days).
- Experimental timeline

**Baseline Blood sample**

**Venous Top-load blood**

**Venous sample at 0, 3 & 24 hours & 2-7 days**

**Conclusion:**
- After intravenous infusion oxygen (PFC), there is no significant change in platelet number and function.
- The result of quantitative observation of platelets is corresponded with the results of coagulation factor analysis.
- Therefore, intravenous infusion with Oxygen will not cause massive or severe coagulopathy.
Effect of Perfluorocarbon on Platelet Number and Function after Intravenous Infusion in Sheep

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Background. Perfluorocarbon emulsions (PFC) can treat traumatic injuries in the battlefield by enhanced delivery of oxygen. A possible side effect of PFC may be thrombocytopenia (in 30~50%) on days 2~5 after intravenous treatment. It is necessary to investigate this phenomenon to exclude platelet inflammatory/embolic safety risks before clinical trial. Methods. Total 24 healthy juvenile sheep (25-30 kg) were randomly divided into 3 groups (n=8/group) with a top load intravenous infusion with either PFC (Oxygent, 60%, 3 g/kg), Hespan (6% hetastarch), or naïve/saline control (naïve =4, saline=4). Venous blood was sampled before the treatment (baseline) and at 0 minute, 3 and 24 hours, 4 and 7 days after infusion and were measured for platelet count, fibrinogen, clot formation time, ADP aggregation & CD62p, etc. Platelet activation was quantitatively observed with scanning electron microscopy (SEM). Results. Comparing baseline with other time points, there were no significant differences on platelet count among control, PFC and Hespan group (435.92±89.42; 391.15±46.60; 437.16±33.63; unit 1000/dl, mean±SE at 4 day post infusion); and fibrinogen level (197.00±20.59; 291.38±79.36; 218.13±25.95; unit mg/dl at 4 day post infusion). Clot time, clot forming time and platelet activation assay (CD62p, %) were not increased compared with baseline or among groups. Morphologically, semi or full-activated platelets (%) were not significantly changed among groups (p>0.05). Conclusion. Intravenous infusion with Oxygent in healthy sheep did not cause significant reduction in number of platelets nor change their activation. Therefore, intravenous infusion with Oxygent will not cause massive or severe coagulopathy. This work was supported by U.S. Army Medical Research and Materiel Command (W81XWH-13-1-0017)

Abstract for Military Health System Research Symposium (MHSRS)
https://mhsrs.amedd.army.mil (register is needed)

2014 Abstract Submission (Due on April 4, 2014 at 5:00 PM ET, notifications will be sent Mid-May 2014)
Abstracts must be no more than 300 words (2000 characters including spaces) and contain a Background, Methods, Results and Conclusion section.
In control group (naïve, no fluid infusion), platelet number showed a less change (±10%). Platelet number was shown a decrease right after 5 ml/kg fluid infusion ((oxygen & perftoran or hespan) at 0 time point and return back at 3 hour time point (Figure 1 & 2).
Changes of Fibrinogen Level after PFC (Oxygent and Perftoran) in Normal Sheep

Fibrinogen level was reduced after fluid infusion (5 ml/kg) in perftoran group at 0 time point (30%) and maintained a stable level till 7 day. For oxygent group, fibrinogen level increased at 24 hour time point till 7 day time point (20% compared with its baseline). In hespan and naïve groups, fibrinogen level changed in about 10% (Figure 3 & 4).
2. Behavioral Observation data after top-load with Oxygent (1st pfc)

During the day (12 hours), the time (%) sheep spent for daily activities of standing up, eating and laying down at different time points (BL=baseline; Top-load = the date with the treatment; and post treatment at 24 hours, 96 hours and 7 days).

We did not record all the experimental cases because video camera system was not completed installed at beginning of the project and had to be relocated after the flood.

![Comparison of the time of standing up in the day](image1)

**Fig 2-1.** Showing the standing up time (%) during the day. Statistical analysis is ongoing.

![Comparison of the time of eating in the day](image2)

**Fig 2-2.** Showing the eating time (%) during the day. Statistical analysis is ongoing.
Fig 2-3. Showing the laying down time (%) during the day. Statistical analysis is ongoing.
1. Transmission Electron Microscopy (TEM) platelet Picture and quantitative criteria

Figure 1-1 (top) & 2 (lower one). TEM photo is obtained from the samples which was observed using scanning electron microscope. These photos shows platelets at baseline blood samples.
Figure 1-3. Shows activation of platelet (from: H. Suzuki et al. / Thrombosis Research 128 (2011) 552–559). Based on the references, our quantitative criteria for platelet activation are as following:

**Score 0:** unchanged discoid form (fig. 1a) showing the peripheral microtubular coil (MTC) in the equatorial plane (fig.1b) or in the cross section (fig. 1c)

**Score 1:** formation of filopodia, and dilatation of the open canalicular system (OCS) (fig. 1d).

**Score 2:** pronounced shape alterations, centralization of the MTC and processing degranulation.

**Score 3:** Degeneration and necrosis. In addition, also the budding and delivery of PMPs.

Another criteria is: Measures of platelet activation on TEM
1. Swollen open canalicular system
2. Spheroid forms with pseudopodia
3. Aggregation/clumping platelets

**Half activation** if either 1 or 2
**Full activation** if swollen OCS (open canalicular system) and pseudopods.
First Step:
Rate: 3mL/Kg/min

MAP drops below 40 mmHg

MAP reaches 50 mmHg or its been 15 min

Reach goal of _____% total blood loss

Second Step:
Rate: 2mL/Kg/min

MAP drops below 35 mmHg

MAP reaches 40 mmHg or its been 15 min

Reach goal of _____% total blood loss

Third Step:
Rate: 1mL/Kg/min

MAP drops below 25 mmHg

MAP reaches 30 mmHg or its been 15 min

Stop when it has been 1 hour since beginning hemorrhage

Reach goal of _____% total blood loss

Shock: 1 hour

Begin Resuscitation with ________ until MAP reaches 60 mmHg and stays stable
HM5 For one of these samples
3. Platelet number and fibrinogen measurement after hemorrhagic shock with oxygent resuscitation, n=2 for each group.

**Fig 3-1.** Platelet count at different time points after hemorrhagic shock (loss 40~50% of total blood), resuscitated with minimum amount of non-blood fluid (hespan + saline or oxygent). N=2 for each group. Platelet number is reduced after resuscitation and first 24 hours, then gradually returns back to baseline. Control group is surgical control with no hemorrhage.

**Fig 3-2.** Fibrinogen level was decreased in hemorrhagic groups on the experimental day and returned back to baseline after 24 hours. The level is higher than the baseline level at 96 hours and 7 days.
Sterile Kits: (each kit has to have an indicator strip on the inside and one on the outside, and Labeled)

How to fold:

Surgeon #1

- 1 Retractor (Weitlaner-Locktite)
- 1 Scalpel Handle
- 1 large curved Scissors (Metzenbaum)
- 1 90º Hemostat
- 6 Curved Hemostats
- 1 Small bulldog clamp
- 1 Larger bulldog clamp
- 1 Inducer
- 1 set of small spring scissors
- 1 small curved scissors
- 2 sharp forceps
- 1 curved forcep (small)
- 1 tissue forcep with teeth
- 1 tissue forcep with ridges

In Surgical Pack
- Drape for surgery
- extra drape to place on top of the tray
- gauze 4x4 and 2x2
- (6-8 pieces) 0-3 silk
- Autoclave strip

Surgeon #2

- 1 Retractor (Weitlaner-Locktite)
- 1 Scalpel Handle
- 1 large curved Scissors (Metzenbaum)
- 6 Curved Hemostats
- 1 Small bulldog clamp
- 1 Inducer
- 1 set of small spring scissors
- 1 small curved scissors
- 2 sharp forceps
- 1 curved forcep (small)
- 1 tissue forcep with teeth
- 1 tissue forcep with ridges

In Surgical Pack
- Drape for surgery
- extra drape to place on top of the tray
- gauze 4x4 and 2x2
- (6-8 pieces) 0-3 silk
- Autoclave strip

Gauze

Beakers

Drapes

Catheters
5. Renovation of Laboratories and the large blast simulator device

Biochemistry laboratory (August 2014)

Large blast simulator laboratory (August 2014)
Move in the new renovated Animal Study Suit on B3, Sanger Hall

Preparing Room (or Rodent Lab)

Large Blast Device Lab
Biochemistry Lab

Large Animal Surgical Room