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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. 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 Oxygent (PFC) in hemorrhagic shock sheep did not cause massive or severe coagulopathy.

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

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Table of Contents

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-O_2** 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. **Oxygent**, another resuscitation product, contains perflubron emulsion (60%, w/v) and has an O_2 carrying capacity similar to 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 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.

2014 Animal use

Quarter Period	Sheep ordered	Top-load model	Hemorrhagic shock	Sum of Used	Model development /death
I	8	8	2	10	2
II	9	3	8	11	3
III	12	3	7	10	7*
IV	8	1	8	9	0
Total	37	15	25	40	12

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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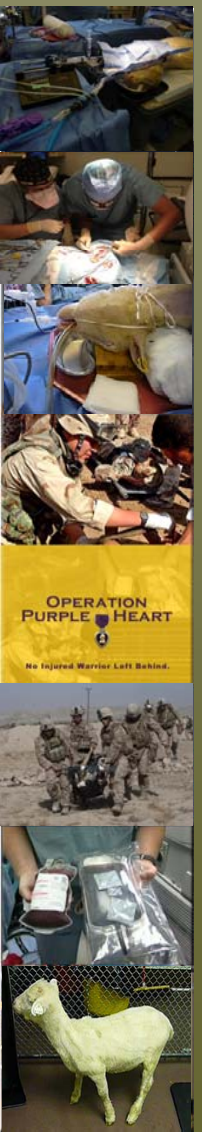


Abstract

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 intravenous treatment. It is necessary to investigate this phenomenon to exclude platelet inflammatory/embolic safety risks before clinical trial. ***Methods:*** A total 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 measurement. ***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 coagulopathy.

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 shows 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), stroke, etc..
- 9 TBI patients were treated with PFC in MCVH with good outcome (Drs. Spiess/Bullock, 2006) .
- PFC may be related with thrombocytopenia (in 30~50%) on days 2~5 after intravenous infusion.
- FDA requests investigation of the phenomenon to exclude platelet inflammatory / 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 & 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 (18-32 kg body weight) were used and randomly divided into 3 groups
- PFC group (n=8); Hespan group (n=8); and Control group (naïve n=4; and saline n = 4)
- Animals were given 7 days for acclimation prior to experiment, and daily vital signs are monitored.

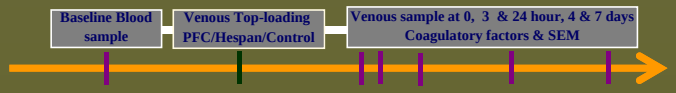
Top-load Procedure & Blood Sample Collection:

- Animal was induction of anesthesia with 5% isoflurane and maintained with 2% isoflurane.
- Animal was 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
- PFC (Oxygent, 60%) or Hespan (6% Hetastarch) was intravenous infusion with 5 ml/kg over 15 minutes.
- Animal was allowed to recover from anesthesia and carried back to DAR facility after Top-load completed.
- Venous blood was collected via jugular puncture at baseline, 0 min, 3 & 24 hour and 4 & 7 day post top-load.

Blood sample measurement & Data analysis:

- Venous samples were measured for coagulatory factors including: platelet count, fibrinogen, clot formation time, ADP aggregation & CD62p, etc..
- Venous 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

Experimental timeline



Results

Morphology of platelet observation with scanning electron microscopy

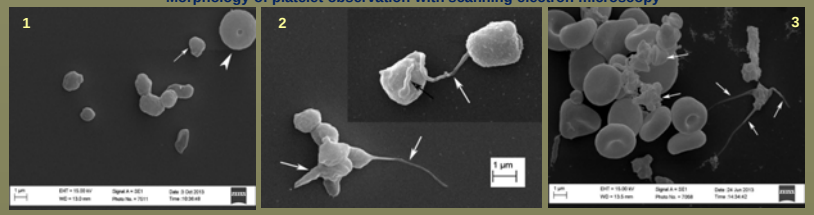
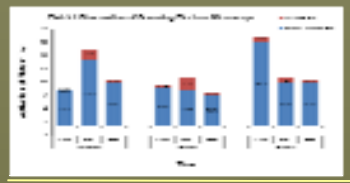


Figure 1: (left) Non-active platelets (white arrow) and red blood cell (arrow head). Non-active platelets are small size with smooth surface.
Figure 2: (middle) Semi-active platelets are with one or 2 processes (white or black arrows) and increase their size.
Figure 3: (right) Active platelets are with 3 or more processes on surface (white or black arrows) and their surface becomes irregular or granular.

Criteria Used to Analyze Platelet Count Images

- Count total number of platelets on image, each sample count 4~ 6 images
- Semi-activated platelet: with 1 or 2 pseudopods
- Full-activated platelet: 3 or more pseudopods or conjugated platelets which groups of platelets that have pseudopods connected
- Non-activated platelet: small size and smooth surface without pseudopods or processes



		Baseline			24 hours			4 days		
		semi-active	active	Total plt count	semi-active	active	Total plt count	semi-active	active	Total plt count
Control	Plt count	99.00	0.00	1203.00	217.00	13.00	2424.00	247.00	13.00	1279.00
n=8	%	8.23	0.00	91.77	8.95	0.54	90.57	19.31	1.02	79.67
Oxygent	Plt count	114.00	91.00	758.00	114.00	48.00	1445.00	102.00	15.00	1058.00
n=8	%	15.04	2.51	82.45	7.89	3.32	88.79	9.64	1.42	88.94
Hespan	Plt count	130.00	4.00	1320.00	145.00	4.00	2005.00	182.00	11.00	1877.00
n=8	%	9.85	0.30	89.85	7.23	0.20	92.57	9.70	0.59	89.72

Figure 4. Percentage of active platelets and semi-active platelets. Cont =control group (n=8); PFC = Oxygent group (n=8); Hes = Hespan group (n=8). Plt = platelet. Platelets were count and calculated how many platelets were active or semi-active (see left table)

Table showed that the total platelets count for each group and semi / full active count as well as percentage of total count.

JMP Statistic Analysis: There are no significant differences in semi or full activated platelet count among groups

Analysis of Variance: Semi-active platelets

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Groups	2	110.5710	55.2855	0.8081	0.4591
Error	21	1436.6286	68.4109		
C. Total	23	1547.1996			

Means and Std Deviations (%): Semi-active platelets

Level	Number	Mean	Std Dev	Lower 95%	Upper 95%
Control	8	9.7975	4.87636	5.7208	13.874
Hespan	8	12.6250	9.65136	4.5563	20.694
Oxygent	8	15.0500	9.39708	7.1938	22.906

Analysis of Variance: full-active platelets

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Groups	2	15.30676	7.65338	1.0058	0.3827
Error	21	159.78624	7.60887		
C. Total	23	175.09300			

Means and Std Deviations (%): full-active platelets

Level	Number	Mean	Std Dev	Lower 95%	Upper 95%
Control	8	0.00000	0.00000	0.000	0.0000
Hespan	8	0.49875	0.93130	-0.280	1.2773
Oxygent	8	1.88750	4.68607	-2.030	5.8052

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Groups	2	104.3187	52.1594	0.7565	0.4817
Error	21	1447.9681	68.9509		
C. Total	23	1552.2868			

Level	Number	Mean	Std Dev	Lower 95%	Upper 95%
Control	8	6.1263	4.8357	2.0835	10.169
Hespan	8	11.0488	11.0378	1.8209	20.277
Oxygent	8	7.4100	7.8509	0.8465	13.973

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Groups	2	175.4440	87.7220	0.9358	0.4080
Error	21	1968.5981	93.7428		
C. Total	23	2144.0422			

Level	Number	Mean	Std Dev	Lower 95%	Upper 95%
Control	8	0.81500	2.2255	-1.046	2.676
Hespan	8	0.09250	0.2616	-0.126	0.311
Oxygent	8	6.15500	16.6195	-7.739	20.049

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Groups	2	386.8232	193.412	1.6294	0.2199
Error	21	2492.7986	118.705		
C. Total	23	2879.6218			

Level	Number	Mean	Std Dev	Lower 95%	Upper 95%
Control	8	16.6763	15.9121	3.3734	29.979
Hespan	8	8.3363	5.2781	3.9237	12.749
Oxygent	8	7.9938	8.6638	0.7506	15.237

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Groups	2	13.19803	6.59902	1.2089	0.3185
Error	21	114.62926	5.45854		
C. Total	23	127.82730			

Level	Number	Mean	Std Dev	Lower 95%	Upper 95%
Control	8	0.45125	0.53996	-0.0002	0.9027
Hespan	8	0.67375	1.27260	-0.390	1.7377
Oxygent	8	2.12375	3.80323	-1.056	5.3033

Platelet Rich Plasma (PRP) preparation:

1. 4.5 ml whole blood in blue tube was centrifuge 165g x 20 minutes.
2. Take all supernatant into 10 ml test tube, mixed with 0.1% glutaraldehyde in in 0.1 M cacodylate buffer into supernatant till 10 ml.
3. Centrifuge with 1200g x 15 minutes, remove all supernatant, keep solid residue
4. Add 2.5% glutaraldehyde in 0.1 M cacodylate buffer and re-suspend till 10 ml volume.
5. Process for SEM (in VCU imagine center)
6. Scope: ZEISS EVO 50XVP, Carl Zeiss SMT, Inc., Peabody, MA.

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

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-activate platelets have 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 coagulopathy.

References:

1. Reicheltová Z1, Májek P, Riedel T, Suttnar J, Dyr JE. Simplified platelet sample preparation for SDS-PAGE-based proteomic studies. Proteomics Clin Appl. 2012 Aug;6(7-8):374-81. doi: 10.1002/prca.201100101.
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3. Chandler WL. Microparticle counts in platelet-rich and platelet-free plasma, effect of centrifugation and sample-processingprotocols. Blood Coagul Fibrinolysis. 2013 Mar;24(2):125-32. doi: 10.1097/MBC.0b013e32835a0824.
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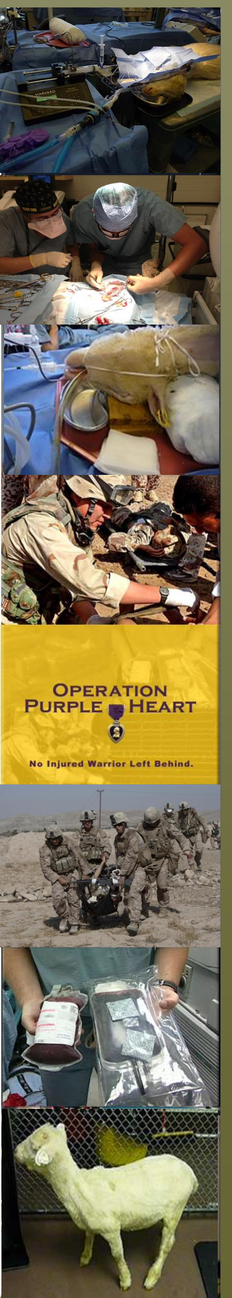
Acknowledgements:

- **VCU School of Medicine Summer Research Fellowship (Mentor: Dr. Jiepei Zhu)**
- **Core laboratories 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-13-1-0017 PI: Dr. Bruce Spiess)**

Effect of Perfluorocarbon on Platelet Number and Function after Intravenous Infusion in Sheep

Jiepei Zhu[1,4], J. Travis. Parsons[2,4], Ph.D., Jacquelyn McCarter[2], Christopher Sweeney[1], J. Mark Hylton, Jr. [1], Erika J. Martin[3,4], Donald Brophy[3,4] and Bruce D. Spiess[1,4],

[1]Departments of Anesthesiology, [2]Neurosurgery, [3]Pharmacology and [4]VCURES- Virginia Commonwealth University Reanimation Engineering Shock Center, 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.
- 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 shows 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), stroke, etc..
- 9 TBI patients were treated with PFC in MCVH with good outcome (Drs. Spiess/Bullock, 2006) .
- PFC may be related with thrombocytopenia (in 30~50%) on days 2~5 after intravenous infusion.
- FDA requests investigation of the phenomenon to exclude platelet inflammatory / 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 & 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 (18-32 kg body weight) were used and randomly divided into 3 groups
- PFC group (n=8); Hespan group (n=8); and 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.

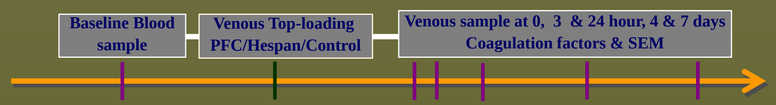
Top-load Procedure & Blood Sample Collection:

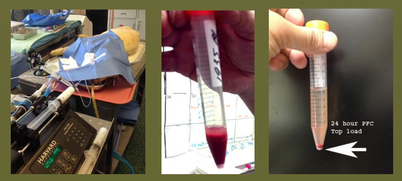
- Animal was induction of anesthesia with 5% isoflurane and maintained with 2% isoflurane during the procedure.
- Animal was intubated (7.5 F) and ventilated (Drager Fabius GS Anesthesia Machine) with 30% O_2; 70% N_2)
- Animal's neck fur was shaved and sanitized; 20 G x 2' needle catheter was puncture into external jugular vein
- PFC (Oxygent, 60%) or Hespan (6% Hetastarch) was intravenous infusion with 5 ml/kg over 15 minutes.
- Animal was allowed to recover from anesthesia and carried back to DAR facility after Top-load completed.
- Venous blood was collected via jugular vein puncture at baseline, 0 min, 3 & 24 hour and 4 & 7 day post top-load.

Blood sample measurement & Data analysis:

- Venous samples were measured for coagulatory factors including:
  - Platelet number count and Alanine aminotransferase (ALT) were measured by VetScan HM5 Hematology system.
  - Fibrinogen was measured using STA Fibrinogen reagent on Diagnostica Stago analyzer.
  - Clotting time, Clot formation time and Clot Angle were measured with Rotem® *delta* (Native, Intrinsic, Extrinsic).
  - Collagen Aggregation, ADP aggregation were measured with 700 Aggregometer.
  - CD62p and vWF Ag (von Willebrand Factor antigen) were measured by Elisa.
- Venous samples collected at 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.

Experimental timeline





Left photo: Anesthetized sheep PFC was receiving PFC infusion with 5 ml/kg (3 g/kg) over 15 minutes.

Middle and Right Photos: Platelet rich plasma preparation. PFC was seen to stay in circulating blood at least for 24 hours post PFC infusion (arrow).

Results

Morphology of platelet observation with scanning electron microscopy

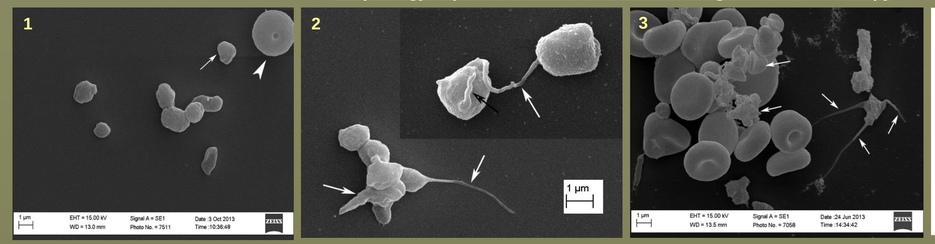
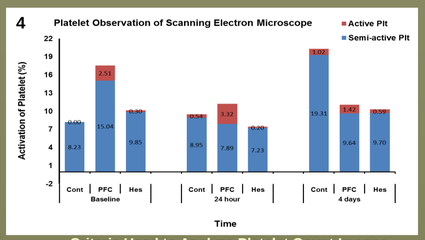
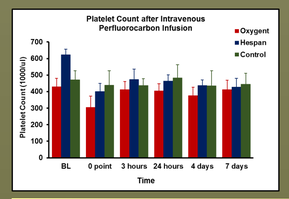


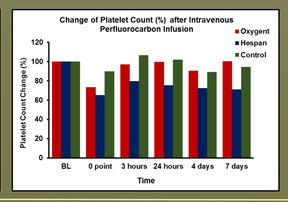
Figure 1: (left) Non-active platelets (white arrow) and red blood cell (arrow head). Non-active platelets are small size with smooth surface.
Figure 2: (middle) Semi-activate platelets are with one or 2 processes (white or black arrows) and increase their size.
Figure 3: (right) Active platelets are with 3 or more processes on surface (white or black arrows) and their surface becomes irregular or granular.
Figure 4. Percentage of active platelets and semi-active platelets. Cont =control group (n=8); PFC = Oxygent group (n=8); Hes = Hespan group (n=8). Plt = platelet. Platelets were count and calculated how many platelets were active or semi-active (see left table)

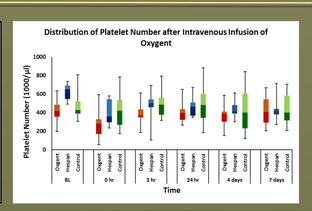


Criteria Used to Analyze Platelet Count Images

- Semi-activated platelet: with 1 or 2 pseudopods
- Full-activated platelet: 3 or more pseudopods or conjugated platelets which groups of platelets that have pseudopods connected
- Non-activated platelet: small size and smooth surface without pseudopods or processes

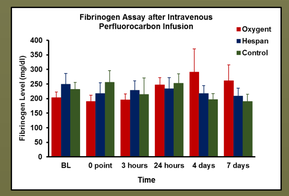


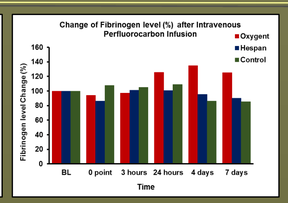


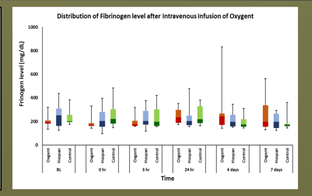


Platelet number: In normal sheep, platelet mean value is about 400,000/µl (range from 100,000 to 800,000). Our study data showed that platelet count did not change significantly when compared with control and Hespan groups (p>0.05)

Left figure: Platelet count and mean distribution with standard error
Middle figure: Percentage change of platelet count after Oxygent infusion.
Right figure: Box plot shows platelet count distribution with minimum value, maximum value, 25%, median and 75% values.

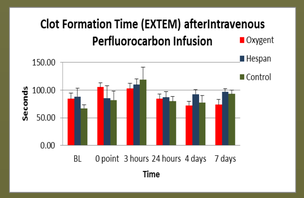




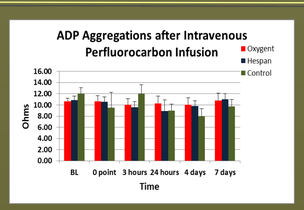


Fibrinogen measurement: There is no significant difference when the groups are compared each other after top-load with Oxygent or Hespan. Also, no significant difference was found within the groups when different time points were compared (p>0.05). Even at day 4, Oxygent group showed a higher fibrinogen measurement (one case), but there is statistically no significant difference.

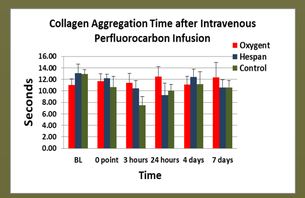
Left figure: Fibrinogen assay and mean distribution with standard error
Middle figure: Percentage change of fibrinogen after Oxygent infusion.
Right figure: Box plot shows fibrinogen level distribution with minimum value, maximum value, 25%, median and 75% values.



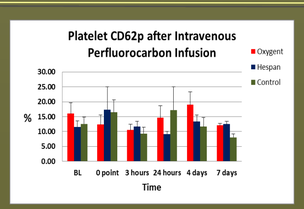
Clot Formation Time (Extem)
There is no significant difference when groups is compared after top-load with Oxygent or Hespan. Also, no significant difference was found within the groups when different time points were compared (p>0.05).



ADP Aggregations
There was no significant difference when groups were compared after top-load with Oxygent or Hespan. Also, no significant difference was found within the groups when different time points were compared (p>0.05).



Collagen Aggregation
There was no significant difference when groups were compared after top-load with Oxygent or Hespan. Also, no significant difference was found within the groups when different time points were compared (p>0.05**).**



CD62p
There was no significant difference when groups were compared after top-load with Oxygent or Hespan. Also, no significant difference was found within the groups when different time points were compared (p>0.05).

Conclusion:

- After intravenous infusion oxygent (PFC), there is no significant change in platelet number and function.
- The result of quantitative observation of platelet is corresponded with the results of coagulation factor analysis.
- Therefore, intravenous infusion with Oxygent will not cause massive or severe coagulopathy.

Acknowledgements:

- Core laboratories 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-13-1-0017 PI: Dr. Bruce Spiess)

Effect of Perfluorocarbon on Platelet Number and Function after Intravenous Infusion in Sheep

Jiepei Zhu[1], J. Travis Parsons[2], Jacquelyn R. McCarter[2], Christopher R. Sweeney[1], Jackson M. Hylton[1], Erika J. Martin[3], Donald Brophy[3] and Bruce D. Spiess[1]
Departments of Anesthesiology[1], Neurosurgery[2], and Pharmacology[3], Virginia Commonwealth University, Richmond VA 23298-0695. ***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 4day 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.

Changes of Platelet Count after PFC (Oxygent & Perftoran) infusion in Normal Sheep

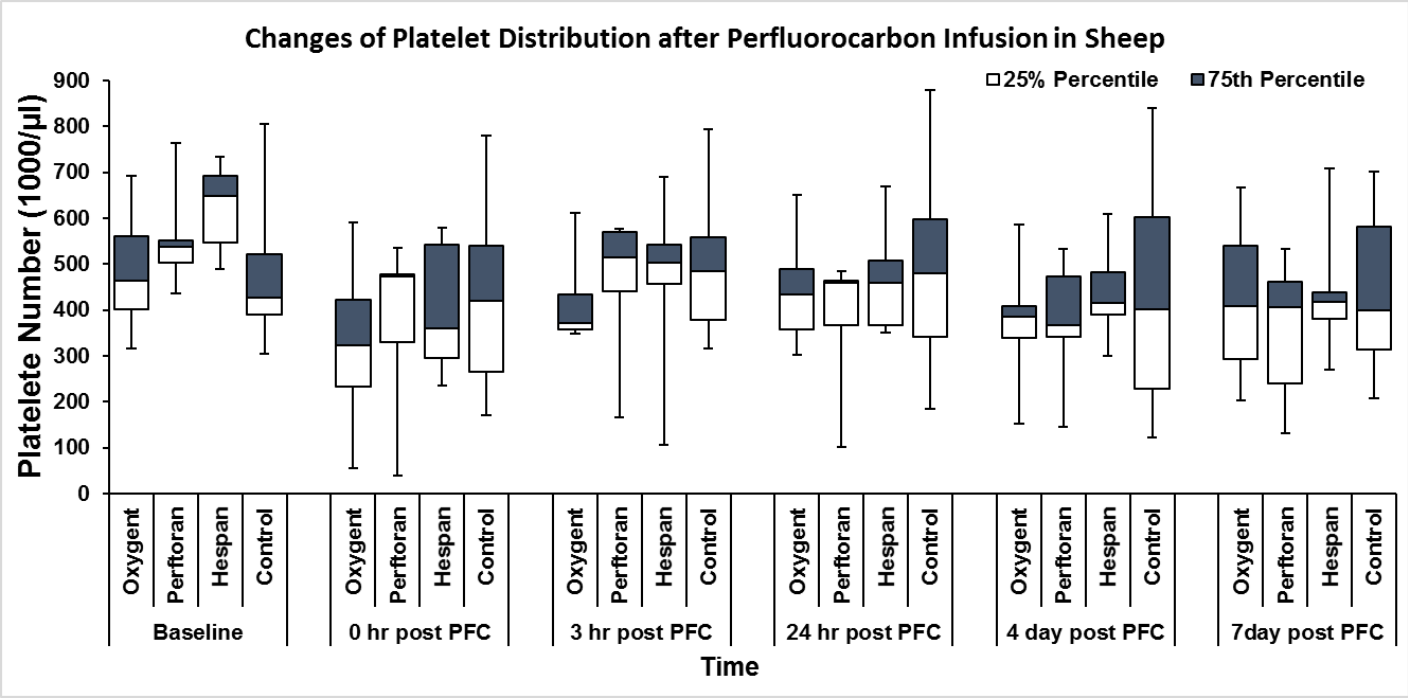


Figure 1.

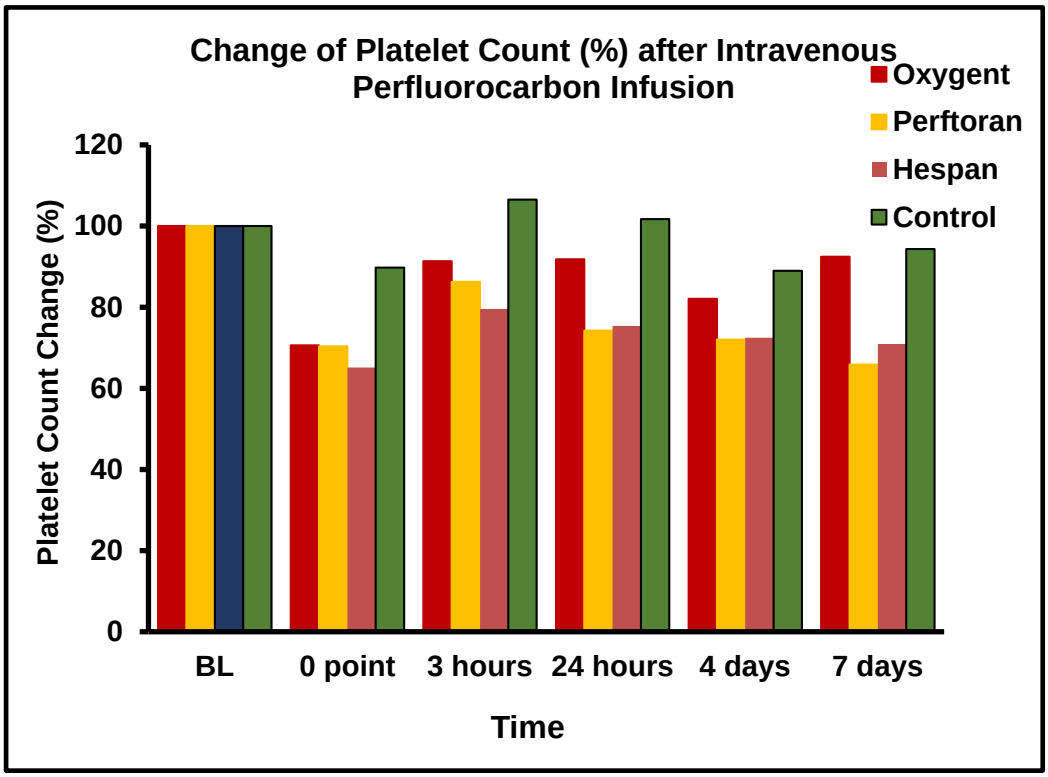


Figure 2.

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

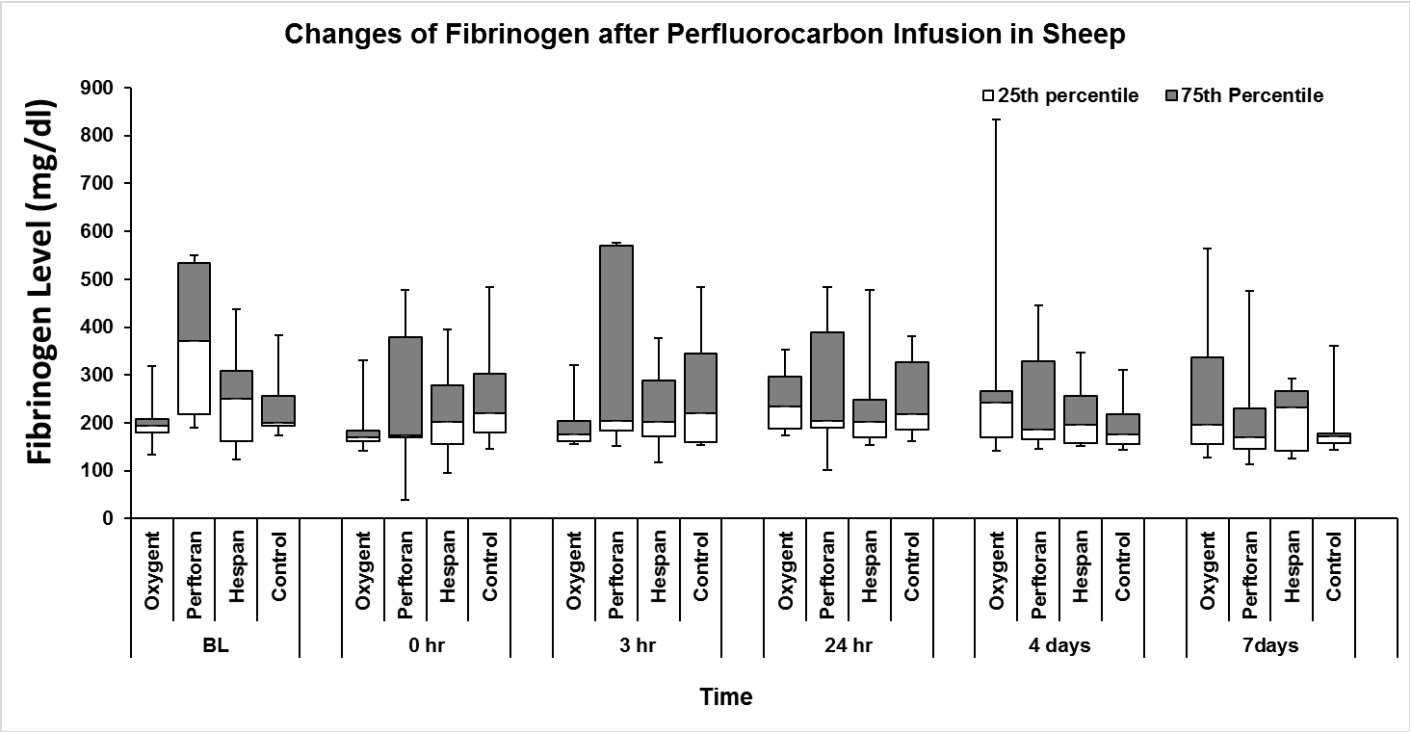


Figure 3.

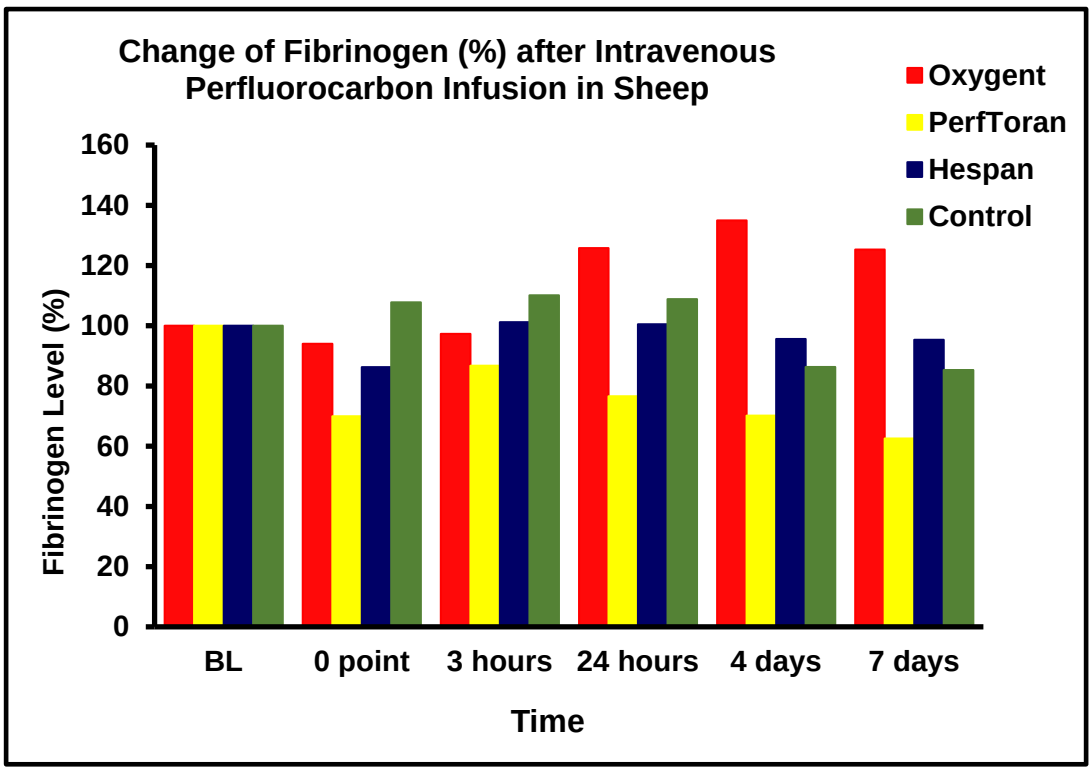


Figure 4

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.

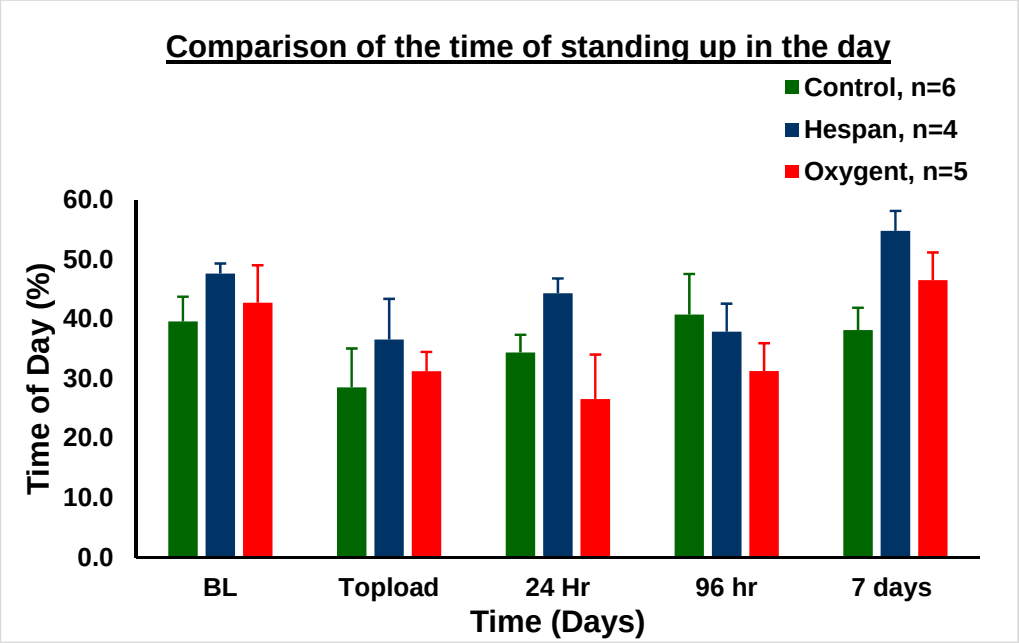


Fig 2-1. Showing the standing up time (%) during the day. Statistical analysis is ongoing.

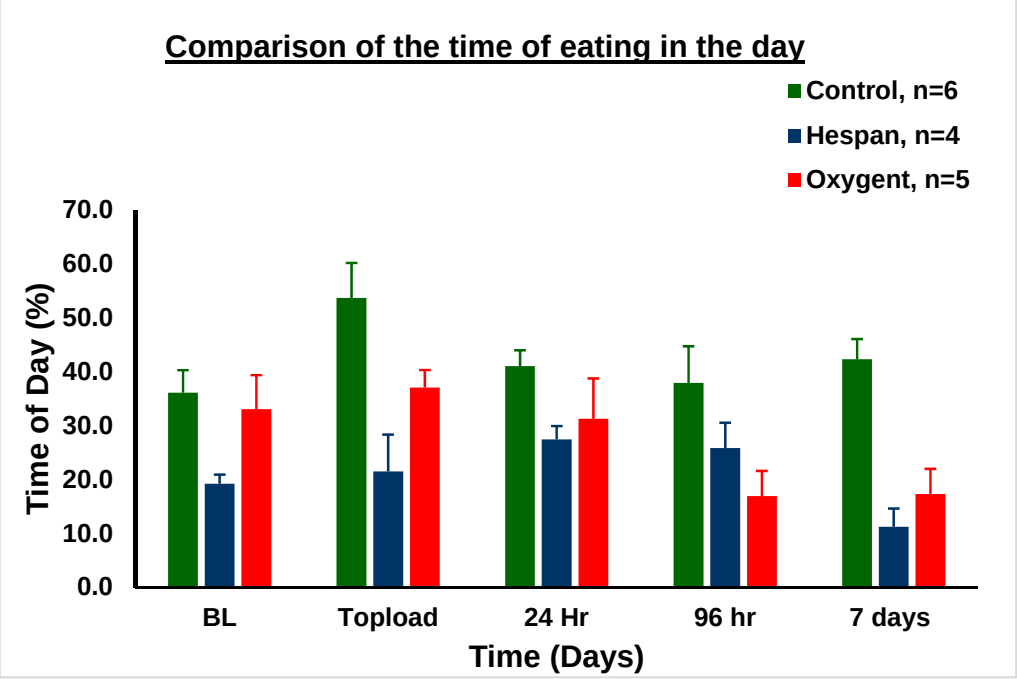


Fig 2-2. Showing the eating time (%) during the day. Statistical analysis is ongoing.

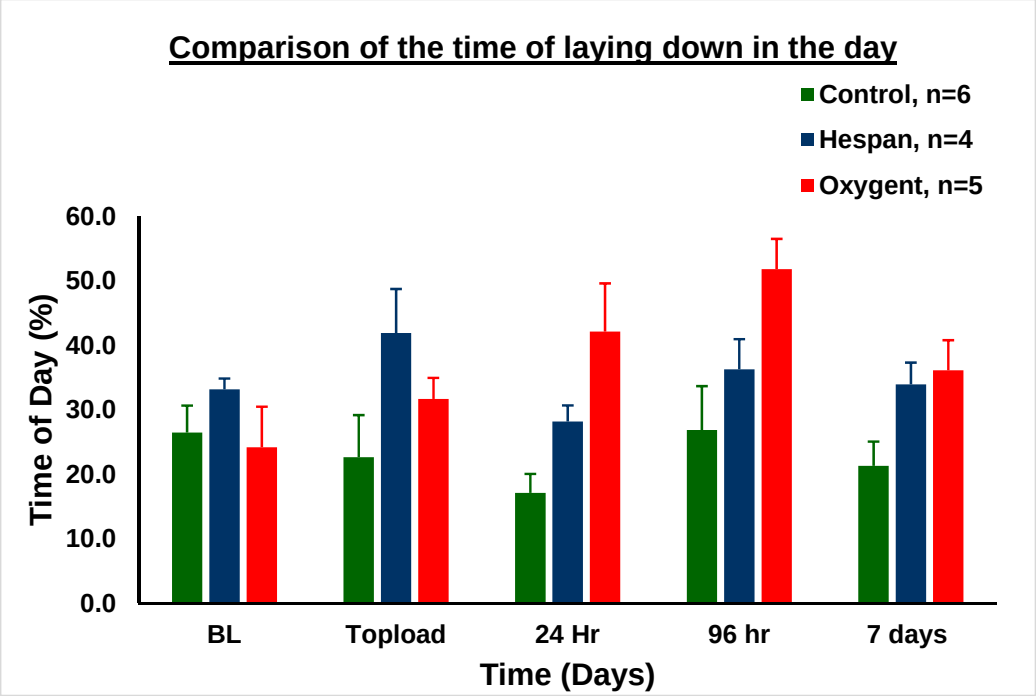


Fig 2-3. Showing the laying down time (%) during the day. Statistical analysis is ongoing.

1. **Transmission Electron Microscopy (TEM) platelet Picture and quantitative criteria**

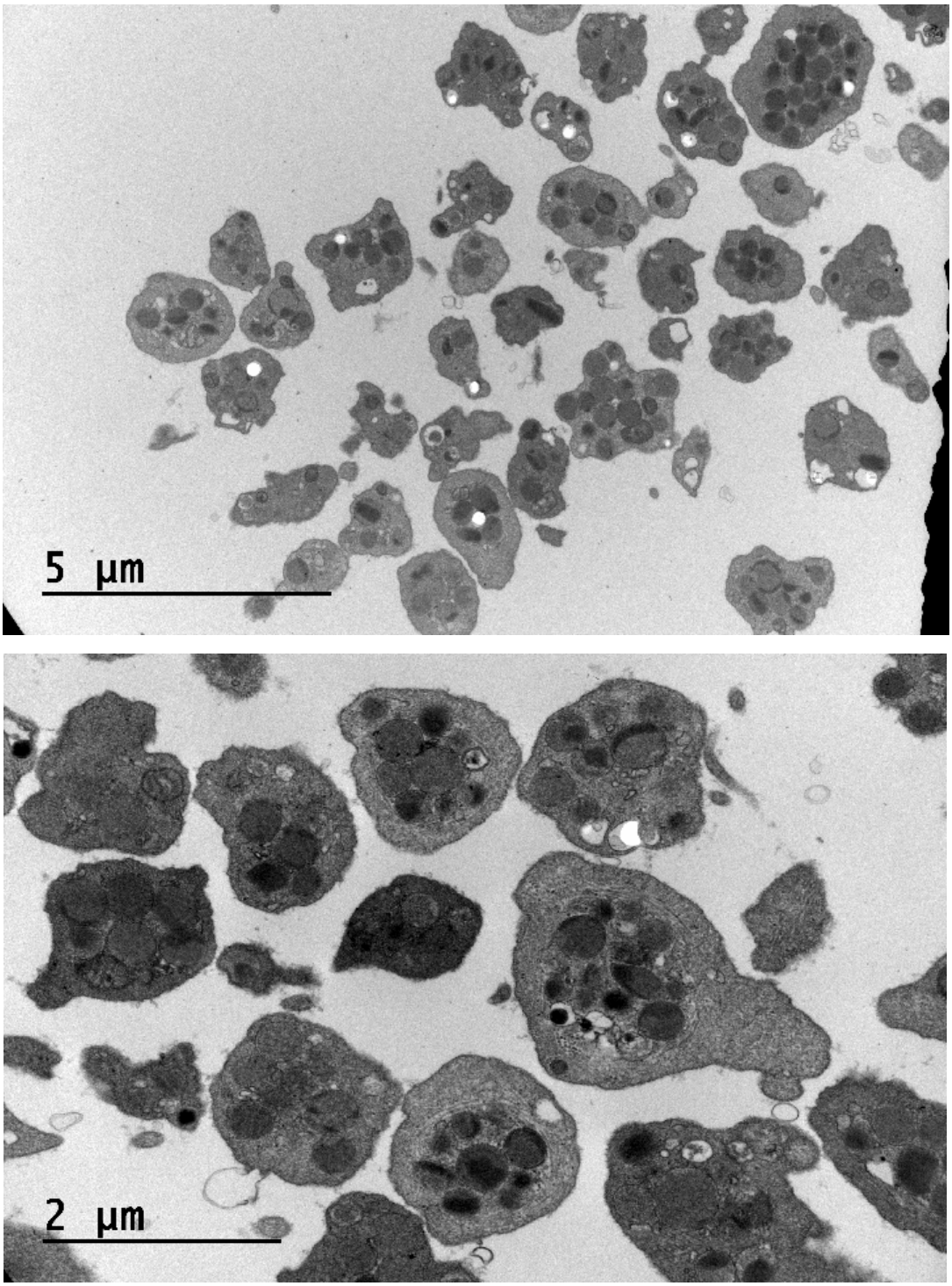


Figure 1-1 (top) & 2 (lower one). TEM photo is obtained from the samples which was observed using scanning electron microscope. These photos shows platelets at baseline blood samples.

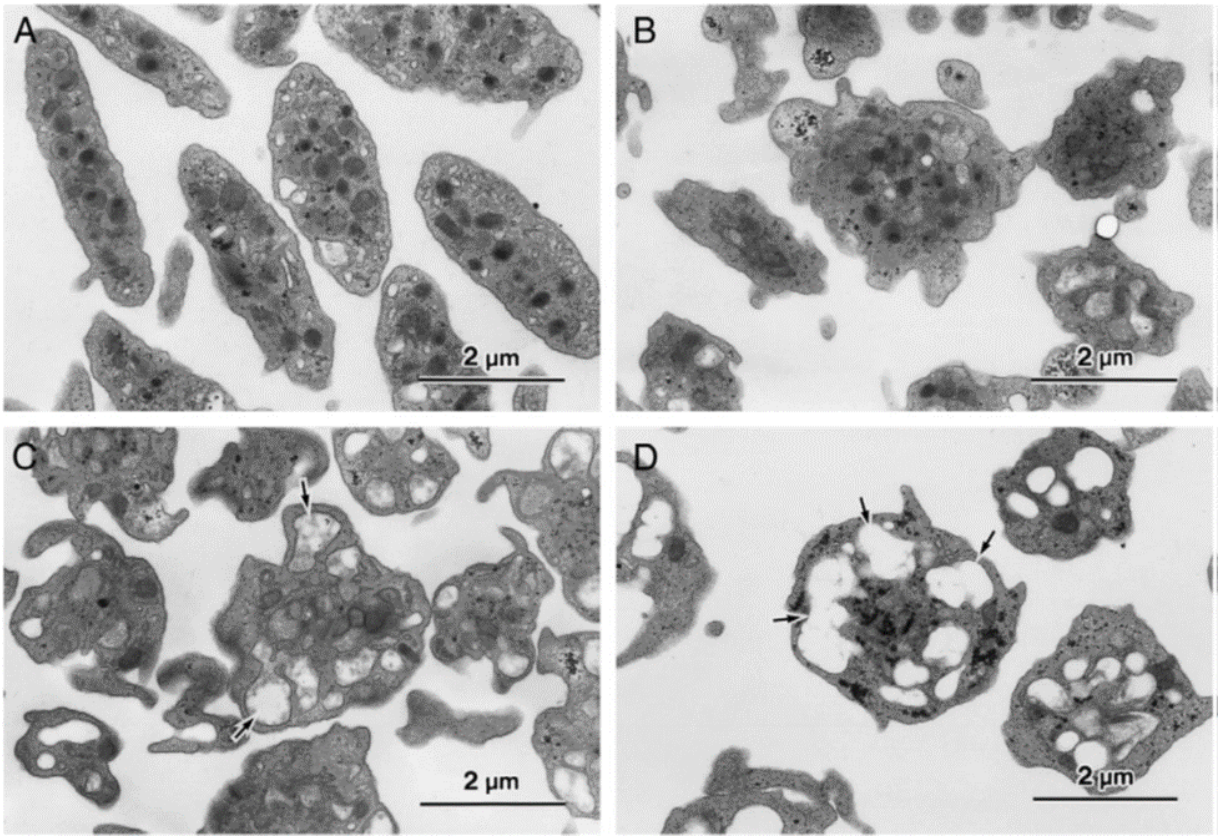


Figure 1-3. Shows activation of platelet (from: H. Suzuki et al. / Thrombosis Research 128 (2011) 552–559). Based on the references, our quantitative criteria for platelet activation are as following:

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).

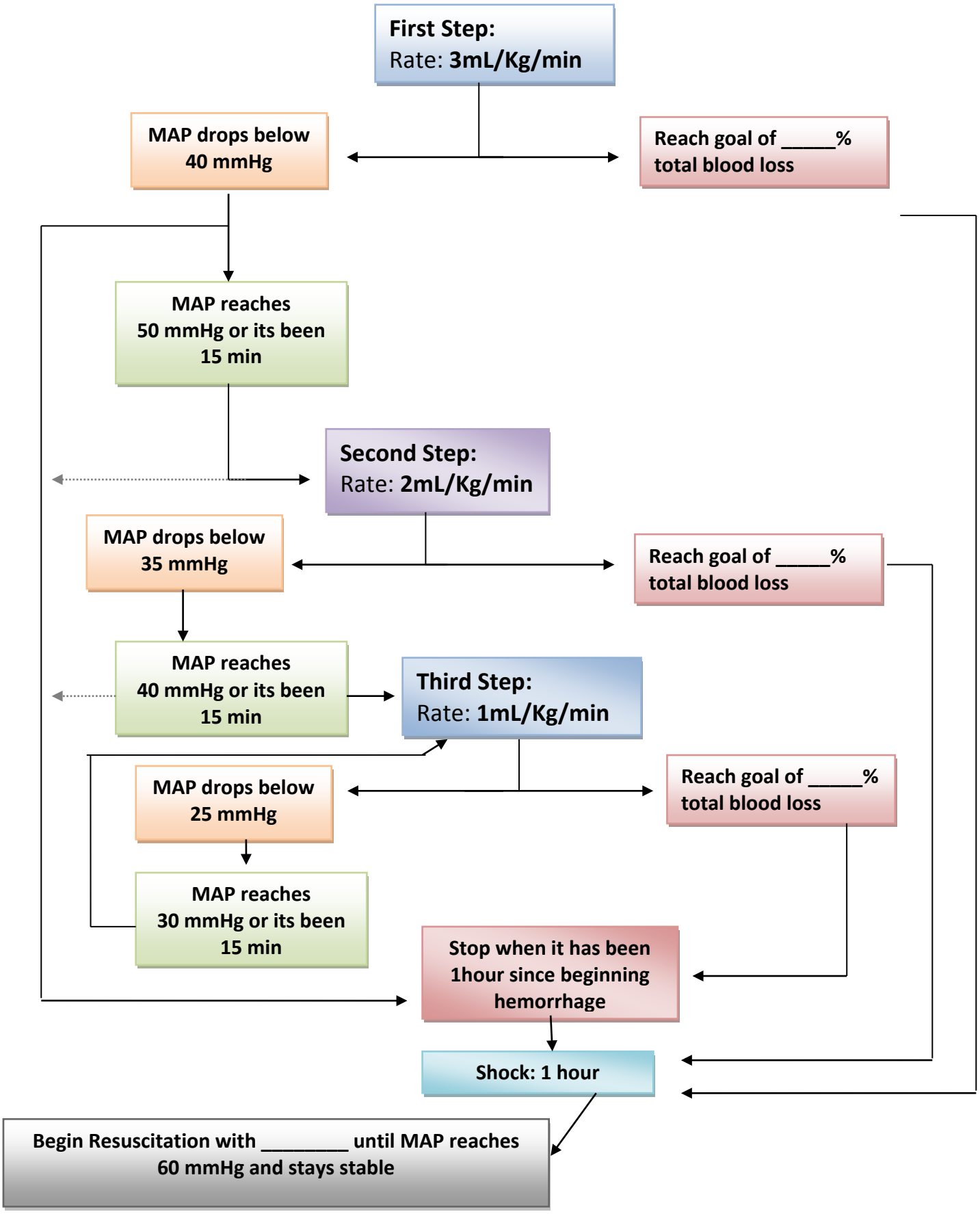
Score 2: pronounced shape alterations, centralization of the MTC and processing degranulation.

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

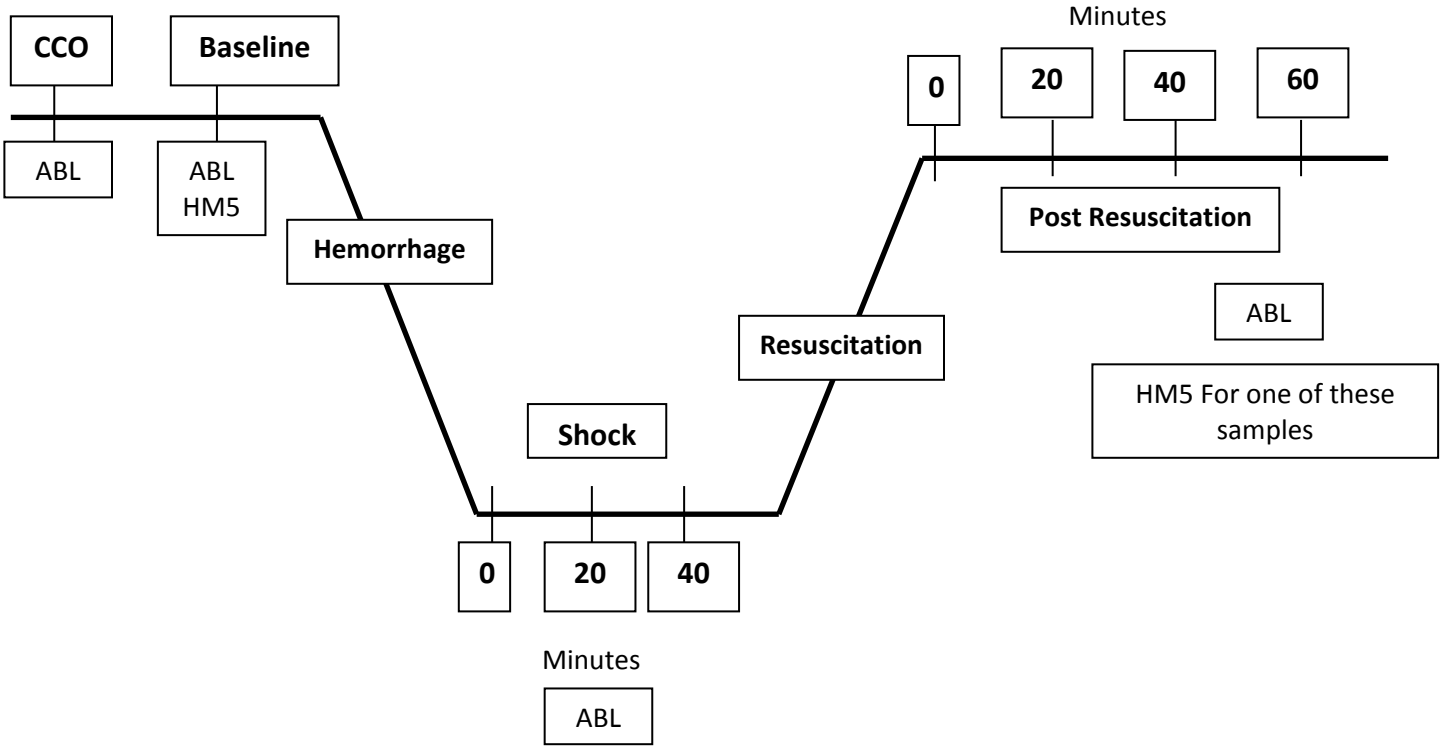
Another criteria is: Measures of platelet activation on TEM
1. Swollen open canalicular system
2. Spheroid forms with pseudopodia
3. Aggregation/clumping platelets

Half activation if either 1 or 2
Full activation if swollen OCS (open canalicular system) and pseudopods.

Index: Hemorrhage Flow Chart



Index: Blood Sample Chart



3. **Platelet number and fibrinogen measurement after hemorrhagic shock with oxygent resuscitation, n=2 for each group.**

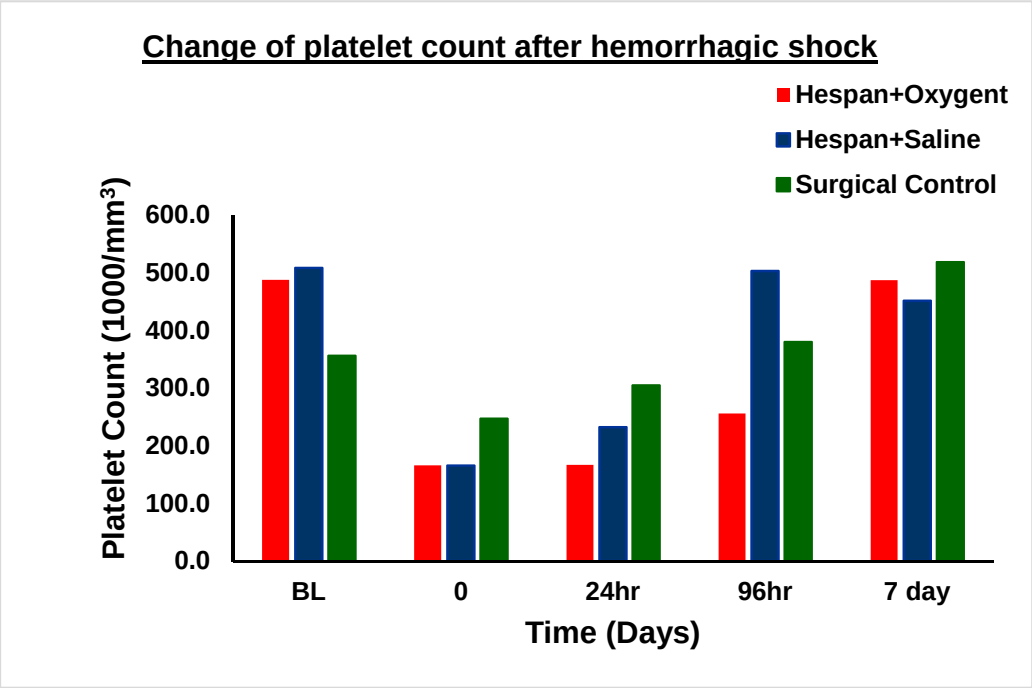


Fig 3-1. Platelet count at different time points after hemorrhagic shock (loss 40~50% of total blood), resuscitated with minimum amount of non-blood fluid (hespan + saline or oxygent). N=2 for each group. Platelet number is reduced after resuscitation and first 24 hours, then gradually returns back to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

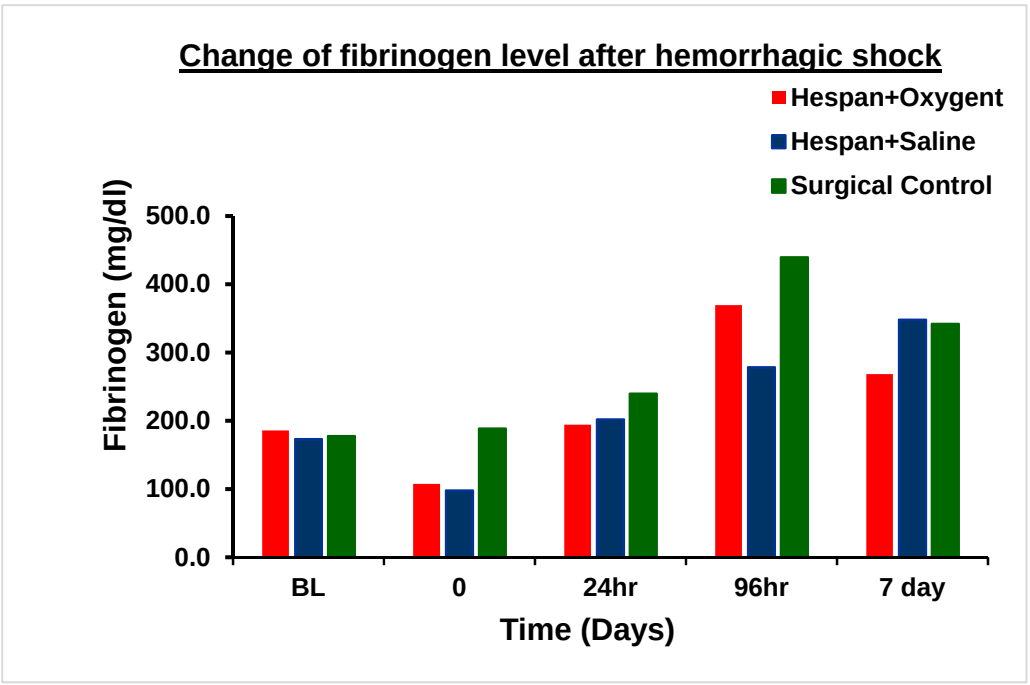
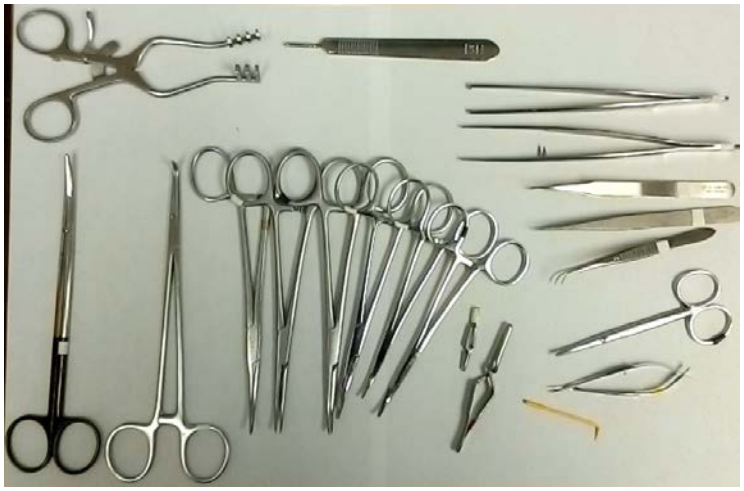


Fig 3-2. Fibrinogen level was decreased in hemorrhagic groups on the experimental day and returned back to baseline after 24 hours. The level is higher than the baseline level at 96 hours and 7 days.

Index: Sterile Technique

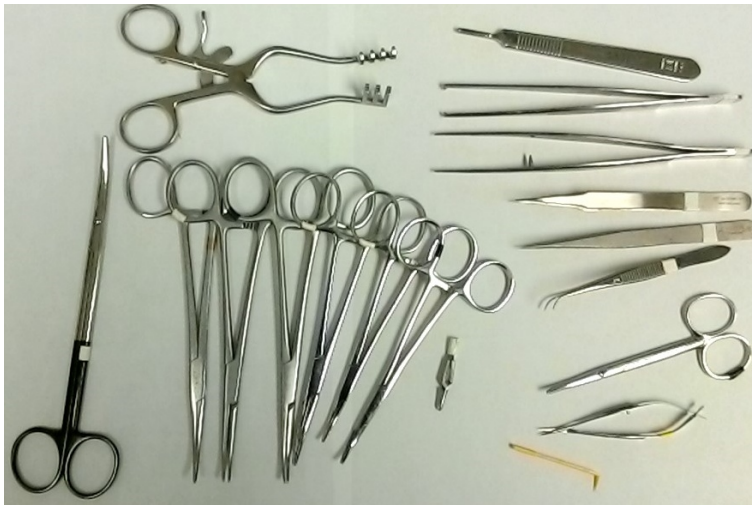
Sterile Kits: (each kit has to have an indicator strip on the inside and one on the outside, and Labeled)
How to fold:
Surgeon #1



- 1 Retractor (Weitlaner-Locktite)
- 1 Scalpel Handle
- 1 large curved Scissors (Metzenbaum)
- 1 90° 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