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14. ABSTRACT
Injured soldiers are surviving in greater numbers than in past conflicts and medical techniques are improving survival. However, some of these soldiers may experience prolonged evacuation times, therefore requiring prolonged field care (PFC). Blood loss (hemorrhage) due to trauma continue to be a concern for combat troops. Prior research suggests that platelets stored at low temperatures (cold-stored) may be useful in bleeding emergencies. Certain compounds (such as tranexamic acid, TXA) have been successful when used within 1h after injury but are unknown to be effective in PFC environments or after resuscitation fluid administration. We proposed to develop a model of trauma and prolonged hemorrhagic shock (HS) to investigate whether cold-stored platelets help to form clots to stop hemorrhage and save lives. If successful, this will help test the efficacy of early versus late injections of promising compounds (such as TXA) that have been shown to reduce mortality in bleeding patients. Using proven, but novel techniques to induce thrombus (clot) formation during HS, platelet adhesion/function was measured in bleeding vessels while monitoring systemic coagulation status of trauma/HS animals for up to 5 hours. Data suggest that refrigerated platelets may be used for hemostasis after trauma and prolonged HS, but when combined with saline resuscitation reduces thrombus formation. This model allows for testing of novel therapeutic interventions to improve coagulation and save lives during PFC.

15. SUBJECT TERMS
Refrigerated platelets; Hemorrhagic shock; Prolonged field care; Hemorrhage; Tranexamic acid; Microcirculation; Cremaster

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TABLE OF CONTENTS

1. Introduction

Background and Goals

Coagulopathy has been observed in trauma patients within the prehospital environment, upon hospital arrival, and may last several hours. Therefore, therapeutic options must be available within the first hour but also at later stages. We aimed to develop a method to evaluate the effect of different administrations (1h and 3h) of tranexamic acid (TXA) on the adhesion of cold stored platelets to damaged blood vessels of normal and traumatized rats. In vivo measurements using intravital confocal videomicroscopy and multiple systemic parameters including coagulation functional tests will be performed with and without resuscitation with different fluids to evaluate the best combination of compounds and injection times in a pre-hospital setting.

Project Design

We first had to establish a model of prolonged hemorrhagic shock. It was then necessary to combine this model with a state-of-the-art, quantitative approach to assess the hemostatic effectiveness of refrigerated platelets at different times during prolong hemorrhagic shock, after trauma. Finally, the hemostatic effectiveness of treatments during prolonged hemorrhagic shock with or without fluid resuscitation could be tested.

To eventually determine the effects of TXA at different time points (1h and 3h), multiple critical steps need to be successfully achieved regarding the quantification of adhesion of cold stored platelets to the endothelial wall of damaged blood vessels after laser puncture. To visualize and measure platelet adhesion *in vivo,* intravital confocal videomicroscopy was the methodology selected, as described is our previous work (Torres Filho et al. - J Thromb Haemost 15(1):163-175, 2017). Systemic parameters including coagulation functional tests (such as thromboelastometry and prothrombin time) were also recorded after fluid resuscitation. As detailed below, experiments were designed to collect data on control (normovolemic) animals, implement a trauma/hemorrhagic shock model, and collect data on hemorrhaged animals treated with saline. All animals were anesthetized:

1) Normal rats (not hemorrhaged – normovolemic)
2) Trauma/Hemorrhagic shock rats
3) Hemorrhagic shock rats resuscitated with normal saline (NS)

2. Keywords

Refrigerated platelets
Hemorrhagic shock
Prolonged field care
Hemorrhage
Tranexamic acid
Microcirculation
Cremaster

3. Accomplishments

Animal Protocol

To perform the experiments, an Animal Use Protocol citing about 90 selected scientific references and involving animals separated into several experimental groups was prepared and submitted to the local (USAISR) IACUC (institutional animal care and use committee). The protocol included a wide range of methodologies and techniques encompassing animal handling, anesthesia and physiological monitoring, microsurgery, confocal intravital videomicroscopy, FACS (fluorescence activated cell sorting), ROTEM (rotational thromboelastometry), cell separation and labeling, ELISA (enzyme-linked immunosorbent assay), computerized laser micro ablation and digital image analysis. The protocol was approved by the IACUC and, in the following years, followed all requirements dictated by the IACUC and all Periodic Review Reports have been approved.

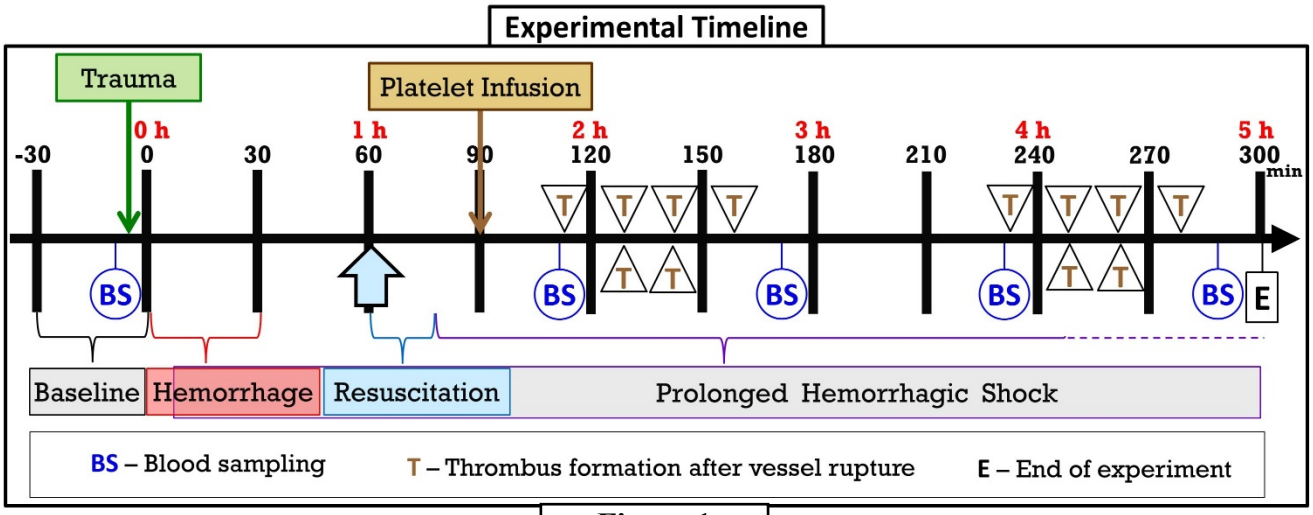

Figure 1

Model Optimization

The experimental portion of the project was based on the timeline design depicted in **Figure 1**. In anesthetized, instrumented animals, we worked on a judicious selection of hemorrhage volumes (to prevent excessive bleeding leading to very early death) and precise blood sampling volumes to measure just the most critical parameters (biomarkers) for monitoring coagulopathy. While systemic parameters were simultaneously recorded, a thrombosis model and a bleeding model were developed depending on the severity of blood vessel wall injury (**Figure 2**). In each model, a computer-controlled laser coupled to the microscope induces an injury into the microvessel wall to which a thrombus is formed while blood continues to flow. The bleeding model, leading to vessel wall rupture, was found to be most relevant, and was used in most experiments. After adjustment of blood sampling volumes, replacement fluids and hemorrhage volumes, the establishment of this model of trauma and prolonged hemorrhagic shock (that includes extensive systemic monitoring and platelet infusion) was successful, and it was possible to obtain measurements for up to 5 hours after start of hemorrhage.

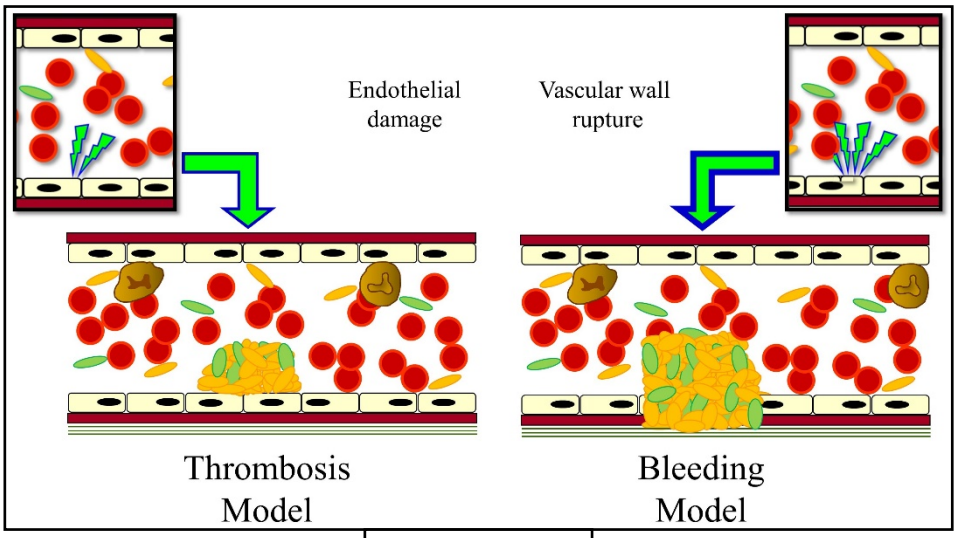

Figure 2

Evaluation of platelet function and thrombus production in vivo. Effect of saline.

Several vessels were studied (ruptured) in each experiment. We used a quantitative evaluation on the ability to produce thrombi in vivo after vessel rupture using laser. The adhesion of platelets with consequent thrombus formation was recorded. Cold-stored fluorescent platelets were previously injected, as indicated by green elliptical cells in **Figures 2** and **3**. Hence, it was possible to measure the fluorescence rise as labeled platelets

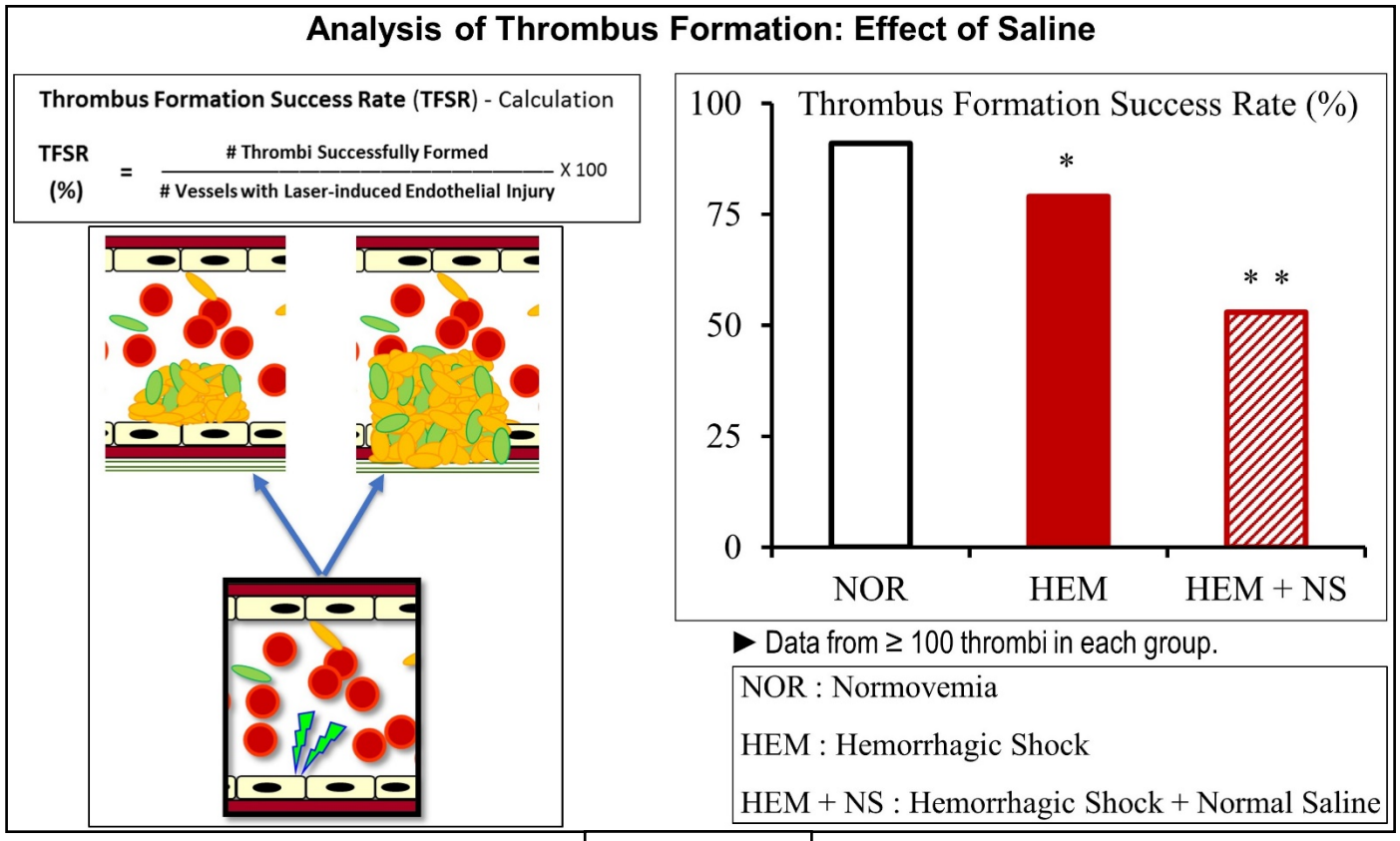

Figure 3

adhere to the thrombus as it increases in size, using off-line image analysis. We also determined the success of thrombus formation by counting the number of thrombi formed relative to the total number of ruptured vessels (**Figure 3**).

New data were collected and analyzed according to new methodology with respect to the use of resuscitation fluids such as saline after trauma and prolonged hemorrhagic shock. The results show that thrombus formation in vivo was reduced by 76% after hemorrhage and further reduced to nearly 50% by resuscitation using saline (**Figure 3**). Details of these findings can be found on the publications listed below and in the Appendices.

Conclusions

We conclude that, in this project, we successfully implemented a quantitative approach to assess hemostatic effectiveness of refrigerated platelets and efficacy of treatments (such as TXA) after trauma and prolonged hemorrhagic shock with or without fluid resuscitation. To show feasibility of the model and state-of-the-art methodology, we measured multiple systemic parameters and demonstrated changes in platelet adhesion after trauma, prolonged hemorrhagic shock and saline resuscitation treatment which are conditions frequently found in the battlefield. The results suggest that refrigerated platelets can be used as hemostatic agents after trauma and during prolonged hemorrhagic shock, as they effectively formed thrombi under these conditions in our model. The outcome of this effort ultimately delivered a relevant model of trauma to test future novel therapeutics for the treatment of shock and hemorrhage in a prolonged care environment. This work will continue to inform future requirements and refined the knowledge and importance of the combined use of TXA and varying resuscitation fluid strategies.

Abstracts and Presentations

Published Abstract:

van Nispen, JA, Bologna, CR, Barraza, D, Hildreth, K, Williams, CE, Dubick, MA and Torres Filho, IP – *Measurements of platelet function in vivo using a rat model of prolonged field care*. J Transl Med 16(Suppl 3):P6, 2018.

Oral Presentation at the XVIIIth Congress of the European Shock Society and IXth Congress of the International Federation of Shock Societies, 2019:

Torres Filho, IP, Barraza, D, Williams, CE, Hildreth, K, Cap, AP and Dubick, MA – *In vivo platelet function and coagulopathy of trauma studied by intravital microscopy: integration of systemic and local parameters*.

Accepted for presentation at the 43rd Annual Conference on Shock, 2020:

Torres Filho, IP, Barraza, D, Williams, CE, Hildreth, K, Cap, AP and Dubick, MA – *Models for studying platelet function and coagulopathy of trauma in vivo.*

Accepted for presentation at the Military Health System Research Symposium (MHSRS), 2020:

Torres Filho, IP, Barraza, D, Williams, CE, Hildreth, K, Cap, AP and Dubick, MA – *Evaluating platelet function and coagulopathy of trauma in vivo using models of combined local and systemic acquisition*.

4. Impact

1. The implemented model mimics military-relevant situations that currently can only be obtained by using anesthetized animals and cannot be fully reproduced using in vitro systems. It allows testing new therapeutic approaches following trauma and prolonged hemorrhagic shock, with or without fluid resuscitation.
2. As an additional benefit of using this system, platelet function/adhesion can be studied under physiological blood flow in fully monitored anesthetized animals after trauma and prolonged hemorrhagic shock.
3. Therefore, progress toward well-defined military requirements such as testing engineered platelets, platelets surrogates, in addition to refrigerated platelets is expected.
4. Since the endothelium is directly investigated in vivo using this method, the system is also useful to test therapeutics and mechanisms involving endotheliopathy of trauma, which has great impact in survival of injured soldiers.

5. Changes/Problems

Several issues were encountered since early in the project. A few months before start, the main Co-investigator that worked with the P.I. for 20 years (not listed in this project but collaborator in the same field) left the laboratory. Around the same time, the senior technician also left the group. Therefore, it was necessary to recruit a new senior technician and start training various specialized techniques. This affected all laboratory activities, including this project. The newly hired technician was less familiar with some specific methods and training required markedly more time.

The trauma / prolonged hemorrhagic shock model implementation was more difficult than anticipated, requiring more experiments than originally designed. The model establishment produced a large amount of useful information to be analyzed from cardio-respiratory parameters, blood biochemistry, and ex-vivo hemostasis in addition to intravital microscopy. This workload, timing and two major additional factors prevented testing of compounds such as TXA. The first factor was the realignment of priorities led by the Leadership in 2019. The second and most important factor was the COVID-19 pandemic, which dramatically limited our resources.

Fortunately, the experiments performed provided enough (and quality) material (on model development, normovolemic, hemorrhaged and saline animals) to reach critically important project goals.

6. Products

Major deliverables for this proposal are *knowledge products*.

The completed work detailed in this report provides advances to the hemostatic care research field and can be used as a platform in further studies on platelets, platelet-surrogates, and new products entering the market in the critical field of coagulopathy of trauma. The ultimate goal of these therapeutics is to reduce morbidity and mortality to trauma / hemorrhagic shock.

7. Participants

PI: Ivo Torres Filho, MD, PhD, FAPS
Co-Investigator: Michael Adam Meledeo, PhD
Technicians: Charnae Williams, BS, David Barraza, MS, Kim Hildreth, MS

8. Special Reporting Requirements

Nothing to report

9. Appendices

Single 4-page PDF file with copies of 4 Abstracts (file: CDMRPL-17-0-DM167100-Appendix.PDF).

Appendix 1

P6

Measurements of platelet function in vivo using a rat model of prolonged field care

Johan A. van Nispen[1], Christopher R. Bologna[2], David Barraza[3], Kim E. Hildreth[3], Charnae E. Williams[3], Michael A. Dubick[3], Ivo P. Torres Filho[3]
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Journal of Translational Medicine 2018, **16(Suppl 3):**P6

Background: Despite advancements in care, soldiers continue to experience long extrication times, during which coagulopathy and decreased perfusion occur (1). Consequentially, developing methods to increase survival during times of prolonged field care (PFC) is of the utmost importance. To test the capability of various drugs to improve these variables, a model was developed wherein blood flow and thrombus formation could be quantified during prolonged hemorrhagic shock.
Materials and methods: Rats anesthetized with isoflurane underwent surgery to exteriorize the cremaster muscle. A baseline blood sample was obtained. Then the rats underwent a laparotomy to simulate trauma. The completion of trauma established the beginning of the experiment at time equal to 0 min. At 30 min post-trauma, rats were hemorrhaged (40% of total calculated blood volume) over 30 min. A post-hemorrhage blood sample was collected. The rats were then subjected to 2 mL blood draws each hour for 5 h after the initial blood sample was collected, with the blood being replaced with normal saline. 90 min into the procedure, cold stored (5 days) platelets from donor rats were fluorescently labeled and infused (approximately 10% of endogenous platelet number). At 120 min, a nitrogen laser was used to induce thrombus formation in selected venules of 17–30 μm in diameter, as described previously (2). Using confocal intravital microscopy, thrombus height and area as well as fluorescent platelet adhesion were measured off-line from video recordings. At 240 min, new recordings were made, whenever possible, to measure the same parameters off-line. The rat was euthanized humanely at the 300 min post-trauma. Rotational thromboelastometry was performed using FIBTEM and EXTEM.
Results: Data are reported as mean ± standard deviation. The average height of the thrombus was 11.3 ± 6.0 μm, and the average area was 265 ± 248 μm^2, during hemorrhagic shock. The EXTEM clotting time (CT) at baseline was 41.00 ± 8.86 s, the alpha angle was 81.5° ± 1.03°, and the clot formation time was 41.13 ± 5.03 s. FIBTEM CT was 35.56 ± 7.41 s and the maximum clot firmness was 14.06 ± 2.86 mm.
Conclusions: A rat model can simulate the scenario of an injured soldier during delayed evacuations. Using these data, measurements of systemic coagulation function and platelet function in vivo during times of PFC are being developed which can be used in experiments to determine the effectiveness of treatments to extend survival.

References

1. Jacob, M, Kumar, P: The challenge in management of hemorrhagic shock in trauma. *Medical Journal, Armed Forces India*, 2014; *70*(2):163–169.
2. Torres Filho IP, Torres LN, Valdez C, Salgado C, Cap AP, Dubick MA: Refrigerated platelets stored in whole blood up to 5 days adhere to thrombi formed during hemorrhagic hypotension in rats. *J Throm Haemostasis, 2017;* 15(1):163–175.

Appendix 2

ABSTRACT 16

IN VIVO PLATELET FUNCTION AND COAGULOPATHY OF TRAUMA STUDIED BY INTRAVITAL MICROSCOPY: INTEGRATION OF SYSTEMIC AND LOCAL PARAMETERS

Ivo Torres Filho[1], *David Barraza*[1], *Charnae Williams*[1], *Kim Hildreth*[1], *Andrew Cap*[2], *and Michael Dubick*[1]

[1]*Damage Control Resuscitation, US Army Institute of Surgical Research, JBSA Fort Sam Houston, TX, USA*

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Introduction: Coagulopathy is a severe complication of trauma and hemorrhagic shock (HS). Since better outcomes require restoration of functional coagulation and microvascular systems, it is essential to assess local blood flow, glycocalyx integrity, and endothelial cell / platelet functions. Hemostasis evaluations are often performed *ex-vivo* but *in vivo* techniques such as confocal intravital videomicroscopy (IVM) are available to redefine evaluation criteria of HS therapeutics. These tools change the paradigm of using only traditional systemic parameters (e.g. derived from ROTEM) to the combined use of local parameters to determine blood/vessel dysfunctions and therapeutic efficacy. Here, we report results of using this approach. **Methods:** Anesthetized rats subjected to 40% hemorrhage were resuscitated with normal saline (NS) or received no fluid. Endothelial injury and vessel rupture were laser-induced in cremaster muscle microvessels. Microvascular diameter, blood flow, thrombus formation and platelet adhesion in the developing thrombus were quantified using confocal IVM, while continuously recording systemic physiological parameters (e.g. arterial blood pressure). A thrombus formation success rate was computed for each animal group dividing the number of microvessels with thrombus successfully formed by the number of vessels with endothelial injuries. *Ex-vivo* hemostasis was also evaluated. Glycocalyx degradation was evaluated from plasma syndecan-1 levels and in separate IVM experiments. **Results:** NS partially improved systemic parameters but damage to glycocalyx and coagulation function were noted, suggesting negative effects of hemodilution and reduction in plasma proteins. Thrombus formation rate decreased from 92% in normovolemic rats to 78% in untreated HS animals, and to 52% in HS rats after NS (mean of 53 vessel injuries/group, 5-8 animals/group). Platelet adhesion reduced after hemorrhage and NS, but platelets stored at room temperature and at 4°C performed equally well as measured by IVM and by ROTEM. **Conclusion:** The systemic-local (in vivo) integrated approach allows a better understanding of underlying injury mechanisms *in vivo* and selection of more effective therapeutic strategies to coagulopathy of trauma.

Supported by US Army Medical Research & Materiel Command.

Presented at the XVIIIth Congress of the European Shock Society and IXth Congress of the International Federation of Shock Societies, 2019

Appendix 3

MODELS FOR STUDYING PLATELET FUNCTION AND COAGULOPATHY OF TRAUMA IN VIVO

Ivo P. Torres Filho[1], David Barraza[1], Charnae E. Williams[1], Kim Hildreth[1], Andrew P. Cap[2] and Michael A. Dubick[1]. [1]Hemorrhage Control Resuscitation, US Army Institute of Surgical Research, JBSA Fort Sam Houston, TX and [2]Coagulation and Blood Research Program, US Army Institute of Surgical Research, JBSA Fort Sam Houston, TX

Background: Effective therapeutic strategies to coagulopathy of trauma require restoration of a functional coagulation and microcirculation. Therefore, assessing local blood flow, glycocalyx integrity, and endothelial cell / platelet functions is paramount, but hemostasis evaluations are often performed *ex-vivo*. However, *in vivo* techniques such as confocal intravital videomicroscopy (IVM) may change the paradigm of using only traditional systemic values (from ROTEM, TEG, etc.) to the combined use of local parameters to determine blood/vessel dysfunctions and therapeutic efficacy. Here, we report models that use this approach, in combination with trauma and prolonged hemorrhagic shock (HS).

Methods: Two HS models of isoflurane anesthetized rats were used (n=6-9 rats/group). 1st group: Animals subjected to severe hemorrhage (40%) were resuscitated with normal saline (NS). 2nd group: Animals were subjected to trauma (laparotomy) and prolonged hemorrhagic hypotension. Untreated hemorrhaged animals served as controls in both groups. Endothelial injury and vessel rupture were laser-induced in cremaster muscle microvessels. In all groups, thrombus formation and platelet adhesion in the developing thrombus were computed in 2 ways, while continuously recording systemic physiological parameters (e.g. arterial pressure). First, processing confocal IVM images of thrombi showing adhered fluorescent platelets. Second, dividing the number of microvessels with thrombus successfully formed by the number of vessels with endothelial injuries. Local microvascular diameter and blood flow were measured. *Ex-vivo* hemostasis was also evaluated. Glycocalyx degradation was evaluated from plasma syndecan-1 levels and separate IVM experiments. Local/systemic parameters were collected at baseline and up to 5 h after hemorrhage.

Results: Both models showed reduction in platelet adhesion following hemorrhage. Thrombus formation was 76% in untreated HS animals and was further reduced after resuscitation with NS. Damage to glycocalyx and coagulation parameters was also noted, despite improvement in some systemic physiological parameters. The performance of platelets stored at room temperature and at 4^{o}C, measured by IVM and ROTEM, was equivalent. In many occasions, thrombus formation and platelet adhesion to microvessels during HS were notably more difficult than ROTEM results indicated, suggesting that local factors not fully measured by ROTEM could be involved.

Conclusions: Models combining systemic and *in vivo* local evaluations contribute to a better understanding of platelet function, underlying injury mechanisms and selection of effective therapeutic strategies to coagulopathy of trauma. This integrated, quantitative approach helps evaluate therapies, positively impacting outcomes of civilian and military victims of trauma and prolonged HS.

Support: US Army Medical Research and Development Command.

Accepted for presentation at the 43rd Annual Conference on Shock, 2020

Appendix 4

MHSRS 2020 Abstract

Evaluating Platelet Function and Coagulopathy of Trauma *in vivo* using Models of Combined Local and Systemic Acquisition

Ivo P. Torres Filho, MD, PhD[1], David Barraza, MS[1], Charnae E. Williams, BS[1], Kim Hildreth, MS[1], Andrew P. Cap, MD, PhD[1], Michael A. Dubick, PhD[2]
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Background. Multiple factors in hemostasis are altered after trauma, hemorrhagic shock (HS), and resuscitation, including platelet function. More importantly, mortality and morbidity are increased by coagulopathy of trauma and changes in the microcirculation. Effective therapeutic strategies to coagulopathy of trauma require restoration of a functional coagulation and microcirculation. Therefore, assessing local blood flow, glycocalyx integrity, and endothelial cell / platelet functions is paramount. Unfortunately, hemostasis evaluations are often performed *ex vivo*. However, *in vivo* techniques such as confocal intravital videomicroscopy may change the paradigm of using only traditional systemic values (from ROTEM, TEG, etc.) to the combined use of local parameters to determine blood and vessel dysfunctions and therapeutic efficacy. Here, we report results obtained after successful implementation of models that employ this combined approach, in experiments with trauma and prolonged HS.

Methods. Sprague-Dawley rats (254–300 g) were anesthetized with isoflurane in room air. Two HS models were implemented. At least six animals per group were studied. In the first group, animals underwent severe hemorrhage (40%) only and were then resuscitated with normal saline. In the second group, animals had first a laparotomy (to simulate battlefield trauma) and then a prolonged hemorrhagic hypotension (to simulate prolonged evacuation, unable to fully restore baseline arterial pressure). Untreated hemorrhaged animals served as controls in both groups. Endothelial injury and vessel rupture were laser-induced in cremaster muscle microvessels. In all groups, thrombus formation and platelet adhesion in the developing thrombus were computed in two ways, while continuously recording systemic physiological parameters (arterial pressure, respiratory rate, lactate, etc.). First, processing confocal intravital videomicroscopy images of thrombi showing adhered fluorescent platelets. Second, dividing the number of microvessels with thrombus successfully formed by the number of vessels with endothelial injuries. Local microvascular diameter and blood flow were measured. *Ex vivo* hemostasis was also evaluated. Glycocalyx degradation was evaluated from plasma syndecan-1 levels and separate intravital videomicroscopy experiments. Local and systemic parameters were collected at baseline and up to 5 h after hemorrhage.

Results. Platelet adhesion was significantly reduced *in vivo* following hemorrhage in both models. In untreated HS animals, thrombus formation was 76% (compared to baseline) and was further reduced after resuscitation with normal saline. Damage to glycocalyx and coagulation parameters was also noted, despite improvement in some systemic physiological parameters. The performance of platelets stored at room temperature and at 4°C, measured by intravital videomicroscopy and ROTEM, was equivalent. In many occasions, thrombus formation and platelet adhesion to microvessels during HS were notably more difficult than ROTEM results indicated, suggesting that local factors not fully measured by ROTEM could be involved.

Conclusions. The combined evaluation of local and systemic parameters achieved in both models, contribute to a better understanding of platelet function, as well as the underlying injury mechanisms and selection of effective therapeutic strategies to coagulopathy of trauma. These models offer critical complementary information to *ex vivo* evaluations that only partially mimic the events occurring *in vivo*. The integrated, quantitative approach helps evaluate therapies, positively impacting outcomes of military and civilian victims of trauma and prolonged HS.

Support: US Army Medical Research and Development Command.

Accepted for presentation at the Military Health System Research Symposium (MHSRS), 2020