Published in final edited form as:

J Trauma Acute Care Surg. 2017 March ; 82(3): 461–469. doi:10.1097/TA.0000000000001343.

Nonhuman primate model of polytraumatic hemorrhagic shock recapitulates early platelet dysfunction observed following severe injury in humans

Leasha J. Schaub, MS, Hunter B. Moore, MD, Andrew P. Cap, MD, PhD, Jacob J. Glaser, MD, Ernest E. Moore, MD, and Forest R. Sheppard, MD

Naval Medical Research Unit San Antonio (L.J.S., J.J.G., F.R.S.), JBSA-Fort Sam Houston, Texas; Department of Surgery (H.B.M., E.E.M.), Denver Health Medical Center, Denver, Colorado; University of Colorado Denver (H.B.M., E.E.M.), Aurora, Colorado; and US Army Institute of Surgical Research (A.P.C.), JBSA-Fort Sam Houston, Texas

Abstract

BACKGROUND—Platelet dysfunction has been described as an early component of trauma-induced coagulopathy. The platelet component of trauma-induced coagulopathy remains to be fully elucidated and translatable animal models are required to facilitate mechanistic investigations. We sought to determine if the early platelet dysfunction described in trauma patients could be recapitulated in a nonhuman primate model of polytraumatic hemorrhagic shock.

METHODS—Twenty-four male rhesus macaques weighting 7 to 14 kg were subjected to 60 minutes (min) of severe pressure-targeted controlled hemorrhagic shock (HS) with and without other injuries. After 60 min, resuscitation with 0.9% NaCl and whole blood was initiated. Platelet counts and platelet aggregation assays were performed at baseline (BSLN), end of shock (EOS; $T = 60$ min), end of resuscitation (EOR; $T = 180$ min), and $T = 360$ min on overall cohort. Results

Address for reprints: Forest R. Sheppard, MD, FACS, CDR, MC, USN, Naval Medical Research Unit San Antonio, Department of Expeditionary and Trauma Medicine, 3650 Chambers Pass, Building 3160, JBSA-Fort Sam Houston, TX 78234; forest.r.sheppard.mil@mail.mil.

This paper was presented at the 75th annual meeting of the American Association for the Surgery of Trauma, September 14–17, 2016, in Waikoloa, Hawaii.

AUTHORSHIP

L.J.S., implemented the study, interpreted data, and drafted and critically revised the manuscript. H.B.M., interpreted data and drafted and critically revised the manuscript. A.P.C., interpreted data and critically revised the manuscript. J.J.G., interpreted data, drafted and critically revised the manuscript. E.E.M., interpreted data, drafted and critically revised the manuscript. F.R.S., principal investigator, was responsible for study conception and design, implementation of study, completion of study, interpretation of data, manuscript drafting, and critical revision.

DISCLOSURE

The authors declare no conflicts of interest.

Declaration of Interest: The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of the Army, Department of Defense, nor the US Government. The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the “Guide for the Care and Use of Laboratory Animals,” Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. This work was supported by funding from the MRMC JPC-6, work unit number G1403. Leasha J. Schaub is contractor of the US Government, Dr. Hunter B. Moore and Dr. Ernest E. Moore are employees of Denver Health Medical Center and University of Colorado, Denver, and LTC Andrew P. Cap, LCDR Jacob J. Glaser, and CDR Forest R. Sheppard are military service members. This work was prepared as part of their official duties. Title 17 USC §105 provides that “copyright protection under this title is not available for any work of the US Government.” Title 17 USC §101 defines a US Government work as a work prepared by a military service member or employee of the US Government as part of that person’s official duties.

are reported as mean $\pm$ standard deviation (SD) or median (interquartile range). Statistical analysis was conducted using Spearman correlation, one-way analysis of variance, two-way repeated-measures analysis of variance, paired *t*-test or Wilcoxon nonparametric test, with *p* < 0.05 considered significant.

RESULTS—Platelet count in all injury cohorts decreased over time, but no animals developed thrombocytopenia. Correlations were observed between platelet aggregation and platelet count for all agonists: adenosine diphosphate, thrombin recognition-activating peptide-6, collagen, and arachidonic acid. Overall, compared to BSLN, platelet aggregation decreased for all agonist at EOS, EOR, and *T* = 360 min. When normalized to platelet count, platelet aggregation in response to agonist thrombin recognition-activating peptide-6 demonstrated no change from BSLN at subsequent time points. Aggregation to adenosine diphosphate was significantly less at EOR but not EOS or *T* = 360 min compared to BSLN. Platelet aggregation to collagen and arachidonic acid was not significantly different at EOS compared to BSLN but was significantly less at EOR and *T* = 360 min.

CONCLUSION—Nonhuman primates manifest early platelet dysfunction in response to polytraumatic hemorrhagic shock, consistent with that reported in severely injured human patients. Nonhuman primate models potentially are translationally valuable for understanding the mechanisms and pathophysiology of trauma-induced platelet dysfunction.

Keywords

Trauma; hemorrhage; platelets; nonhuman primate

Hemorrhagic shock (HS) remains the leading cause of preventable trauma-related deaths in civilian and military populations.^{1,2} The combination of severe injury and hypotension initiates a cascade of biologic events that disrupt hemostasis, in a process termed trauma-induced coagulopathy (TIC).³ The presence of TIC on admission to the emergency department is associated with a four-fold increased mortality risk, increased blood product utilization, and organ failure.^{4–6} Trauma-induced coagulopathy is a multifactorial process that disrupts hemostasis through impairments in coagulation, fibrinolysis, and platelet function.⁷

Platelet dysfunction independent of platelet count has been described as an early component in the pathophysiology of TIC.^{8–10} In the context of the cell-based model of hemostasis, platelets play a pivotal role in maintaining hemostasis following trauma through adhesion and aggregation to establish a hemostatic plug at the site of vessel injury and membrane amplification of coagulation protease activation of thrombus formation.¹¹ Platelet dysfunction can cause near-total impairment of clot formation even in the presence of normal-range platelet counts.¹²

The specific mechanisms driving platelet dysfunction in TIC remain to be fully elucidated, and translatable animal models are needed to develop therapeutic strategies to attenuate the pathogenesis of these coagulation changes. Multiple animal models have been developed to study mechanisms of trauma and HS and to evaluate therapeutic agents; however, clinical translatability remains questionable owing to hematologic and physiologic disparities

associated with low-order species such as rodents^{13,14} and xenogeneic complications seen in swine.^{15–17} Nonhuman primates (NHP) are orthologous to humans physiologically, immunologically, and genetically, making them ideal for human disease modeling.^{17–19} Furthermore, old-world monkeys (i.e., rhesus macaques) circumvent xenogeneic reactions that disrupt the evaluation of human-derived blood products in lower-order species.^{15–17} We sought to determine if the early platelet dysfunction described in trauma patients could be recapitulated in an NHP model of polytraumatic HS, thereby expanding our understanding of the platelet pathophysiology and uncovering opportunities for subsequent investigations and treatment development.

METHODS

Ethical Approval and Accreditation

The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the 711th Human Performance Wing, Joint Base San Antonio-Fort Sam Houston, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 2011. All procedures were performed in facilities accredited by the Association for Assessment and Accreditation for Laboratory Animal Care International. This manuscript focuses on one distinct study aim of a multifaceted polytraumatic HS NHP protocol.

Preoperative Preparation

Rhesus macaques used in this study were housed in compliance with the Secretary of the Navy Instruction (SECNAVINST) 3900.38C regulations. Feed was withheld 12 hours before surgery. Twenty-four male rhesus macaques weighing 7 to 14 kg and aged 5 to 11 years were randomized into three injury cohorts (n = 8/cohort): 60-minute severe pressure-targeted controlled HS; HS + soft tissue injury (HS-ST); or HS-ST + musculoskeletal injury (HS-ST-MS), while an additional three male rhesus macaques were used as anesthesia controls (n = 3).

Anesthesia and Analgesia

Nonhuman primates were sedated with Telazol (3.0 mg/kg; Fort Dodge Animal Health, Overland Park, KS, USA), premedicated with an analgesic (Buprenex, 0.03 mg/kg; Reckitt Benkiser, Slough, Berkshire, UK), and weighed (Detecto Scale, Webb City, MO, USA). Airway intubation was achieved with placement of a pediatric endotracheal tube (size, 4–5.5 mm; Rusch–Teleflex, Research Triangle Park, NC, USA), and placed on a Dräger Apollo Anesthesia Workstation (Dräger Medical Inc, Telford, PA, USA) with volume-controlled respiration (10 mL/kg) at 12 to 15 breaths per minute, FiO₂ of 21% to 25% and isoflurane (1.0–2.0%) inhalational anesthesia. Core body temperature was monitored continuously via 10 Fr rectal temperature probe connected to a Datex Ohmeda CardiCap/5 patient monitor (GE Healthcare Systems, Wauwatosa, WI, USA) and maintained between 36.0°C and 38.0°C.

Arterial and Venous Line Placement

The right femoral artery was cannulated with a SIL-C70 silicone catheter (Solomon Scientific, Skokie, IL, USA) to facilitate blood sampling and invasive, continuous blood pressure monitoring using the Datex Ohmeda CardiCap/5 patient monitor (General Electric Co, Fairfield, CT, USA). The right femoral vein and left femoral artery were cannulated with a 14-gauge large bore catheter (Jorgensen Laboratories, Loveland, CO, USA) for intravenous resuscitation fluid infusion and to facilitate hemorrhage, respectively.

Soft Tissue and Musculoskeletal Injury

After baseline (BSLN) blood samples were drawn, a 10-minute “injury window” was observed in all groups to standardize procedure times. Within this time frame, the randomly assigned additional tissue injury, or no injury, was produced (Fig. 1).

Soft tissue injury was created by a 15-cm laparotomy incision spanning from the xiphoid process toward the umbilicus. The laparotomy incision remained open during the shock period and was closed in two layers (fascia and skin) with continuous sutures during the resuscitation phase.

The musculoskeletal injury was created on the left leg using a longitudinal 5-cm incision spanning the anterior mid-femur to distal femur followed by blunt dissection to separate muscle and muscle insertions from a 4-cm span over the mid-femur. An oscillating power bone saw was used to transect the femur, gauze was placed in the wound for blood collection, and the skin incision was closed with staples. During the resuscitation phase, the gauze was removed and weighed to quantify blood loss, the femur was approximated and fixated with steel plates and screws, and the incision closed in two layers (fascia and skin) with sutures.

Pressure-Targeted Controlled HS

Hemorrhage was initiated by opening a stopcock in-line with the left arterial catheter allowing free flow of blood until the mean arterial pressure reached 20 mm Hg. This moment marked the beginning ($T = 0$ minute) of the shock period, additional blood was withdrawn as needed to maintain a mean arterial pressure of 20 to 24 mm Hg until the end of shock (EOS; $T = 60$ minutes). Hemorrhaged blood was collected in a blood donor bag containing anticoagulant citrate dextrose solution—solution A (ACDA-A) at a 1:10 ratio for subsequent use in resuscitation.

Standardized Resuscitation

At $T = 60$ minutes, blood samples were collected, and animals were then resuscitated at a constant rate with 0.9% NaCl (normal saline, $2 \times$ shed blood volume (SBV)) infused over 30 minutes, followed by whole blood (50% SBV) infused over 30 minutes and then $1 \times$ SBV of NS over 60 minutes. Whole blood transfusion was preceded with 100 mg/mL calcium gluconate (Vedco, Saint Joseph, MO, USA) to elevate possible calcium chelation effects of the anticoagulant. At the end of resuscitation (EOR; $T = 180$ minutes), animals were monitored under anesthesia until $T = 360$ minutes.

Laboratory Analysis

Arterial blood samples were collected at: BSLN, EOS, EOR, and $T = 360$ minutes.

Whole blood analysis was performed for complete blood cell count (HemaTrue; HESKA, Loveland, CO, USA) and arterial blood gas (GEM Premier 4000; Instrumentation Laboratory, Bedford, MA, USA).

Platelet aggregation was performed on whole blood by impedance aggregometry (Multiplate; Verum Diagnostica GmbH, Munich, Germany) using the agonists: adenosine diphosphate (ADP), collagen (COL), thrombin receptor-activating peptide-6 (TRAP) and arachidonic acid (AA). Agonist responses are reported as area under the aggregation curve in units (U) over a 6-minute measurement period and as non-normalized and normalized to platelet count.

Data Reporting and Statistical Analysis

Statistical analyses were performed using Prism 6 (GraphPad Software, Inc, La Jolla, CA, USA). Spearman correlations were used to correlate platelets' responsiveness to agonists with platelet count. For overall cohort analysis, normally distributed variables were expressed as mean $\pm$ standard deviation (SD) and Student t -test used for comparisons, while non-normally distributed variables were expressed as median and interquartile range (IQR) and the Wilcoxon nonparametric test was used for comparisons. For comparison between cohorts, a one-way analysis of variance and two-way repeated-measures analysis of variance were performed. A $p < 0.05$ was considered significant.

RESULTS

Survival, Blood Loss, and Hematological Parameters

Analysis of percent blood loss ($59\% \pm 9\%$ vs $52\% \pm 8\%$ vs $49\% \pm 7\%$ for HS, HS-ST, and HS-ST-MS, respectively) and markers of injury severity at all-time points as determined by lactate, base deficit, pH, bicarbonate anion (HCO_3^-), partial pressure of carbon dioxide, and platelet count (Table 1) revealed no significant differences between the three injury paradigms. Possible effects of citrated whole blood transfusion were tracked with ionized calcium at all time points (BSLN, EOS, EOR, and $T = 360$ minutes). While levels do drop, with the nadir at EOR, by $T = 360$ minutes, levels begin to return to BSLN for the overall cohort (1.04 ± 0.05 vs 0.95 ± 0.07 vs 0.89 ± 0.06 vs 0.95 ± 0.06). It is important to note that our BSLN values started off at less than 1.12 mmol/L, the cutoff for hypocalcemia, but never went below 0.8 mmol/L, a marker for ionized calcium treatment.²⁰ Within the overall study cohort, lactate peaked at 3.1 ± 1.7 mmol/L and troughs were seen for base deficit at -7.5 ± 1.6 mmol/L, pH at 7.39 ± 0.03 , HCO_3^- at 23.4 ± 1.3 mmol/L, partial pressure of carbon dioxide at 35.7 ± 6.4 mm Hg, and platelet count at $200 \pm 35 \times 10^3/\mu\text{L}$. Subsequent analyses were done on the overall study cohort. All NHPs survived to the final time point of $T = 360$ minutes and never became thrombocytopenic (platelet count $< 100 \times 10^3/\mu\text{L}$) (Fig. 2).

Platelet Impedance Aggregometry

When compared to HS, both HS-ST and HS-ST-MS demonstrated decreased aggregation in response to ADP and/or COL at EOS. Platelet aggregation in response to ADP for HS measured at 24.9 U (IQR, 19.8–34.2 U) and decreased by 37% in HS-ST (15.6 U [IQR, 10.0–18.4 U]; $p = 0.0049$) and 38% in HS-ST-MS (15.5 U [IQR, 12.8–23.1 U]; $p = 0.0129$); and in response to COL, HS measured at 25.4 U (IQR, 19.6–33.6 U) and decreased by 35% in HS-ST-MS (16.4 U [IQR, 11.9–19.0 U]; $p = 0.0253$).

In the overall cohort, platelet responsiveness decreased for all agonists over the course of the study compared to BSLN values (Fig. 3). Platelet aggregation in response to ADP was measured at 25.6 U (IQR, 18.6–28.5 U) at BSLN and decreased by 32% at EOS (17.3 U [IQR, 13.5–25.3 U], $p = 0.0244$), 48% at EOR (13.2 U [IQR, 8.5–16.7 U], $p < 0.0001$), and 30% at $T = 360$ minutes (18.0 U [IQR, 12.4–22.6 U], $p = 0.0002$). Platelet aggregation in response to COL (BSLN, 25.8 U [IQR, 21.0–30.4 U]) decreased 27% at EOS (18.9 U [IQR, 13.5–27.3 U], $p = 0.0395$), 60% at EOR (10.3 U [IQR, 6.2–14.8 U], $p < 0.0001$), and 43% at $T = 360$ minutes (14.7 U [IQR, 8.7–17.6 U], $p < 0.0001$). Platelet aggregation in response to TRAP (BSLN, 30.5 U [IQR, 16.2–60.5 U]) decreased 39% at EOS (18.6 U [IQR, 6.0–53.5 U], $p = 0.0149$), 60% at EOR (12.3 U [IQR, 5.3–45.0 U], $p = 0.0006$), and 35% at $T = 360$ minutes (19.9 U [IQR, 10.8–43.7 U], $p = 0.0164$). Platelet aggregation in response to AA (BSLN, 30.0 U [IQR, 15.3–35.6 U]) decreased 32% at EOS (20.5 U [IQR, 13.4–24.9 U], $p = 0.0164$), 93% at EOR (2.1 U [IQR, 1.2–5.6 U], $p < 0.0001$), and 78% at $T = 360$ minutes (6.6 U [2.5–15.3 U], $p < 0.0001$). Anesthesia controls had no significant alterations in platelet aggregation compared to BSLN at all time points in response to agonists (ADP, TRAP, COL, and AA); data not shown. Correlations were observed between platelet response and platelet count for all agonists: ADP ($r = 0.525$, $p < 0.0001$), COL ($r = 0.472$, $p < 0.0001$), TRAP ($r = 0.538$, $p < 0.0001$), and AA ($r = 0.541$, $p < 0.0001$; Fig. 4).

Normalized platelet aggregation data are presented in Figure 5. Platelet responsiveness to the agonist TRAP demonstrated no change from BSLN at subsequent time points.

Responsiveness to ADP was significantly less at EOR but not EOS or $T = 360$ minutes compared to BSLN. Platelet responsiveness to COL and AA was not significantly different at EOS compared to BSLN, however, was significantly less at EOR and $T = 360$ minutes.

DISCUSSION

Platelet dysfunction was observed 60 minutes after initiation of shock involving all platelet activation pathways in this NHP model of HS with tissue injury. These platelet dysfunctions were exacerbated after animals were resuscitated, and function remained significantly depressed up for 180 minutes after resuscitation from shock. While platelet counts progressively decreased during the experiment until resuscitation, none of the animals developed clinically significant thrombocytopenia (e.g., platelet count $< 100,000/\mu\text{L}$), consistent with the concept of a trauma-induced thrombasthenia. While correlations between platelet dysfunction and platelet counts were significant, normalizing platelet function to platelet count demonstrated that platelet dysfunction remained significant for specific pathways at specific time points during the experiment with the exception of the TRAP pathway.

Kutcher et al.⁹ and Solomon et al.,²¹ reported weak correlations between platelet count and responsiveness to agonists ADP, COL, TRAP, and AA and observed platelet dysfunction in the presence of normal platelet counts. While our aggregation data reflect trauma patient observations, the significance and extent of platelet responsiveness to agonists was affected after platelet count normalization. These findings conflict with reports in the literature that suggest platelet counts in the observed range are unlikely to interfere with platelet functional analysis.^{22,23} Both COL and AA showed similar dysfunction at EOR and $T = 360$ minutes when non-normalized or normalized, supporting the notion that both COL and AA have important implications in trauma and have already been identified to be independent predictors of mortality in a risk-adjusted model.⁹ It remains important to appreciate that the timing and partial recovery of platelet dysfunction is not identical between all platelet receptor pathways. These receptors coordinate a complex hemostasis process that dictates the platelets' adhesion, cohesion, degranulation, and contribution to clot formation.²⁴

Up to 92% of critically injured patients during their hospitalization will develop platelet dysfunction.⁹ The ADP pathway was the first platelet dysfunction appreciated in this model that persisted to be significant after adjusting for platelet count. This is consistent with clinical studies by Wohlaer et al.⁸ and Moore et al.,¹⁰ in which ADP dysfunction was appreciated in blood samples obtained before hospital or upon arrival at the emergency department in patients that had received minimal resuscitation. The mechanism driving ADP dysfunction in trauma remains elusive. Cationic metabolites such as succinate have been implicated in early ADP platelet inhibition, but not lactate.¹⁰ This is supported by this study in which the animal's nadir of platelet function was after resuscitation when lactate levels had already normalized. There are numerous metabolic pathways that are clinically unmeasured in humans that begin to demonstrate significant changes within minutes of HS observed in a rat model.²⁵ Although the addition of soft tissue and femur injury caused slight dysfunction at EOS, their addition did not drastically change the pattern, and all injury cohorts demonstrated similar dysfunction by EOR. While our study focused on hemorrhage, soft tissue, and long bone injuries, there may be specific injury patterns, which also cause platelet dysfunction that were not assessed in this model; for example, ADP dysfunction in traumatic brain injury has been found to be prognostic of mortality and not dependent on hypotension.^{26,27}

With respect to normalized data, there was a descending order of effect for agonists ADP, COL, and AA on platelet dysfunction at EOR, with downplay of ADP, and COL and AA demonstrating similar effects between non-normalized and normalized data. All three of these agonists lead to the production of thromboxane A₂, with ADP and COL also modulating other mechanisms of activation, degranulation, and aggregation.^{28,29} These data are suggestive that resuscitation may have some effect on thromboxane A₂ production or receptor binding, with thromboxane A₂ receptor desensitization having been demonstrated to cause platelet dysfunction.^{30,31} In this study, the greatest dysfunction was seen in response to agonist AA, which solely examines the production of thromboxane A₂ through cyclooxygenase 1. Platelet aggregation in response to TRAP remained unchanged when normalized to platelet count and is known to act independent of both thromboxane A₂ and ADP pathways.^{31,32} Specifically, TRAP-induced degranulation is independent of thromboxane A₂ generation, while thromboxane A₂ generation is important for ADP and

COL-induced degranulation.³³ This supports the notion that resuscitation affects some mechanism of the thromboxane A2 pathway. The effects of reduced aggregation to COL and AA on uncontrolled bleeding are still unknown, but this research offers an interesting insight into the role of platelet dysfunction(s) in trauma.

This study demonstrates that platelet dysfunction can be reproduced in an animal model of HS and polytrauma. This reproducible model of platelet dysfunction that persists 180 minutes after resuscitation remains clinically relevant, as return to normal blood pressure does not correct the animal's platelet coagulation abnormality. With recent animal work demonstrating that platelet dysfunction precedes other manifestations of trauma-induced coagulopathy,³⁴ which has been already suggested in humans,¹⁰ it is essential to increase our understanding of the role of platelet dysfunction in trauma. Arguably, the prognostic value of platelet function after minor trauma has no association with adverse outcomes and mortality.³⁵ There may be a protective role in having a moderate level of platelet dysfunction after trauma. However, exacerbation of this dysfunction with the inappropriate resuscitation fluid could be lethal. This has previously been demonstrated when evaluating fibrinolysis in a rodent model in which normal saline exacerbated coagulopathy while plasma resuscitation kept coagulopathy at a similar level and improved survival in a model of HS.³⁶ In our model, we used a NS followed by whole blood resuscitation. The component of the acute coagulopathy of resuscitation represents a promising area of investigation, as therapeutic opportunities may exist to attenuate this reperfusion coagulopathy. Additionally, it is unknown if the shock environment might render transfused platelets dysfunctional; therefore, timing of platelet transfusion for greatest effectiveness should be considered.

While the platelet dysfunction data are consistent with that seen in human trauma, the severity of our model was not. Despite profound hemodynamic shock in these NHPs, lactate levels peaked at 3.1 ± 1.7 mmol/L, and the nadir for base deficit was -7.5 ± 1.6 mmol/L; this relatively mild metabolic effect may have contributed to the milder platelet dysfunction seen at the EOS when compared to EOR. The use of normal saline as a resuscitation fluid could also be a confounding factor possibly causing hyperchloremic acidosis, which may be affecting platelet function independent of the actual shock and injury. Based on this possibility, we are currently undertaking a study to specifically look at the effect of different crystalloids on the metabolic and coagulation effects in these models.

Here, we provide a translatable model for further elucidation of the mechanism of early platelet dysfunction following trauma and for evaluating therapeutics and resuscitation strategies. We have demonstrated that in a model of HS with or without tissue injury, platelet dysfunction can be reproduced and seems to be independent of critically low platelet counts, which are used by many trauma centers to guide platelet transfusion. This animal model demonstrates numerous points of impact for therapeutic potential and investigation of the role of platelet dysfunction in trauma.

DISCUSSION

Dr. Weidun A. Guo (Buffalo, New York) I would like to thank the AAST for the privilege to discuss this well-written manuscript by the Sheppard group. I just learned that Leasha is a pre-med student. Leasha, I compliment you on your excellent presentation.

I congratulate the authors for this forward-leaning translational research to determine the early platelet dysfunction. The study is important from both a research and clinical standpoint, because it provides a non-primate model for further understanding of the mechanisms behind this acute uncontrollable bleeding that accounts for 30–40% of patients who die in the acute phase of trauma.

The followings are my questions to the authors.

My first question is in regard to the importance of the nonhuman primate model in the TIC research. Is there any data demonstrating that a rodent model is not appropriate for TIC? On the other hand, is there any human data available to be compared side-by-side with your results, before claiming that the platelet dysfunction in this non-human primate model is consistent with that in humans?

My second question relates to the body weight of the primates. The Rhesus Macaques weighed 7–14 kilograms in your study. While an adult Rhesus Macaques weighs 5–7 kg on average, was a 14 kg monkey obese or morbidly obese? If this is the case, then how could you make sure that the obesity is not a confounding factor for the coagulation abnormality?

The third question is about the blood transfusion. In your study, the primates received autologous blood which was collected during the induction of hemorrhagic shock, and later transfused back to the same individual. This is in stark contrast to what we are doing in our clinical practice, in which trauma patients receive homologous blood from human donors. Homologous blood transfusion by all means causes more immunologic reaction as compared to autologous transfusion. Could you please comment on the rationale of autologous blood transfusion in your study and the potential clinical implications?

My last question is about the hemorrhagic shock. Perhaps the most striking parameter that I found from your study is the MAP, which was only 20–24 mmHg during hemorrhagic shock. I am wondering if this scenario is translatable to hemorrhagic shock in humans, in which a MAP of 20–24 mmHg for 60 minutes is unlikely compatible with life.

Regardless of the issues raised above, I applaud the authors for their efforts in pushing the horizon and leading the way in the investigation of trauma-induced coagulopathy. I anticipate the authors will provide important future direction for improving outcomes in our patients with trauma-induced coagulopathy.

Thank you.

Dr. Martin Schreiber (Portland, Oregon) That was an outstanding presentation. I was extremely impressed by the number of the rules that the Navy has on your disclosure slide.

My question for you, however, is the following. Your model is extremely aggressive—lots of trauma, lots of bleeding. Is it possible that we're not really actually seeing platelet dysfunction but what we are actually seeing is that you are maximally activating the platelets with your model so that when you do these in vitro tests what you are actually testing is platelets that have already been maximally activated so that they no longer aggregate and they no longer function? Is that a possibility?

Dr. H. Gill Cryer (Los Angeles, California) Was this re-shed blood and, if so, was the platelet function checked? Were there endothelial activation studies on that as well?

Dr. Matthew D. Neal (Pittsburgh, Pennsylvania) I want to congratulate you on an outstanding presentation and your group on the development of a model that's going to help us all in translation from basic bench research to clinical practice.

My question follows directly with Dr. Schreiber's and that is the systemic activation of platelets is a real concern in the setting of severe poly-trauma. And I wonder if what you are measuring in the circulation just represents platelets that are unaffected or subjected to dilution?

Have you looked elsewhere, meaning have you looked at the histopathology? Have you looked for sequestration of platelets within lung or other end organs, suggesting that there may have been deposition or aggregation activation that occurred prior to the point at which you measured?

Ms. Leasha J. Schaub (San Antonio, Texas) Dr. Guo thank you for your critical review of the manuscript and excellent questions. In response to the importance of a non-human primate model of TIC, as briefly discussed, this was a small piece of a larger project, whose purpose was to develop a poly-trauma hemorrhagic shock model for the evaluation of human-derived blood products. With the use of human-derived blood products comes the complication of xenogeneic compatibility that can be seen in lower species, which is why non-human primates were chosen.

As for rodent models of TIC, clinical translatability remains questionable due to hematological and physiological disparities. When considering platelets, rodent's platelets display several ultrastructural differences compared to humans, such as size, number and granularity, while non-human primates are very similar.

In comparison to human data, as I discussed during the results, this data was compared to Kutcher and Cohen's paper from 2012. Through this comparison we were able to demonstrate similar correlations and aggregation responses. We are continuously working with collaborators to compare data from these non-human primate models with those from human studies.

As for the effects of BMI on platelet aggregation, a post-hoc analysis was done examining these effects by grouping non-human primates based on BMI: normal, obese, and morbidly obese. It was found that BMI did not have an effect on platelet aggregation but did, in fact, have an effect on the immune response.

The rationale of using autologous transfusion was that this was considered a best-case scenario; part of which, at least experimentally, involves circumventing variability associated with homologous transfusion. Again, this model was developed to facilitate evaluations of novel life-saving resuscitation fluids that incorporate logistically advantageous, lyophilized human-derived blood products such as plasma and platelets. In this capacity, autologous whole blood represents a best-case scenario or metric by which to gauge the efficacy of various therapeutics.

The target MAP of 20 to 24 millimeter of mercury was based in observations from a previously developed uncontrolled liver hemorrhage model. During that model it was noticed that upon injury induction, non-human primates' MAPs would drop to around 20 millimeter of mercury, this observation was used as a basis for the pressure targeted controlled hemorrhage protocol.

Dr. Schreiber, if what is being tested is the over-activation of platelets instead of platelet dysfunction is a great question. We believe that this is not the case based on what is seen in the hemorrhage alone model, which is simply a free bleed from catheterized femoral artery. So, there is really nowhere for those platelets to aggregate at due to the lack of injury. And with this model we saw very similar responses as in our overall cohort.

Dr. Cryer, yes it was shed blood that was used for reinfusion and no, platelet function was not checked; however, that is an excellent point and something that will be implemented in future studies.

As for endothelial activation markers, yes, we have looked at syndecan-1 and heparan sulfate, and are continuing to work on assessing other markers of endothelial damage, such as endothelin-1.

Dr. Neal, in response to if we are measuring platelets that are unaffected or subjected to dilution, we don't believe we are. If we were measuring unaffected platelets, I would think that similar aggregation would have been seen between baseline and other time points upon activation with agonist. As for a dilution effect, we corrected for that fact by normalizing the aggregation data to platelet count, thereby taking away the effect of platelet count differences.

Histopathology was performed on multiple end organs including, heart, lungs, liver, kidney, and spleen. Sequestration of platelets was not noted by the pathologist in any cases. This is an excellent point and will be implemented in future studies.

Acknowledgments

The authors thank Kassandra Ozuna, Heather Grossman, Amanda Sooter, and Darren Fryer for their excellent technical support.

Disclosure of Funding: This work was supported by funding from Medical Research and Materials Command (MRMC) Joint Program Committee 6 (JPC-6), work unit number G1403.

References

1. Eastridge BJ, Hardin M, Cantrell J, Oetjen-Gerdes L, Zubko T, Mallak C, Wade CE, Simmons J, Mace J, Mabry R, et al. Died of wounds on the battlefield: causation and implications for improving combat casualty care. *J Trauma*. 2011; 71(Suppl 1):S4–S8. [PubMed: 21795876]
2. Pfeifer R, Tarkin IS, Rocos B, Pape HC. Patterns of mortality and causes of death in polytrauma patients—has anything changed? *Injury*. 2009; 40(9):907–911. [PubMed: 19540488]
3. Brohi K, Cohen MJ, Ganter MT, Matthay MA, Mackersie RC, Pittet JF. Acute traumatic coagulopathy: initiated by hypoperfusion: modulated through the protein C pathway? *Ann Surg*. 2007; 245(5):812–818. [PubMed: 17457176]
4. Brohi K, Singh J, Heron M, Coats T. Acute traumatic coagulopathy. *J Trauma*. 2003; 54(6):1127–1130. [PubMed: 12813333]
5. MacLeod JB, Lynn M, McKenney MG, Cohn SM, Murtha M. Early coagulopathy predicts mortality in trauma. *J Trauma*. 2003; 55(1):39–44. [PubMed: 12855879]
6. Maegele M, Lefering R, Yucel N, Tjardes T, Rixen D, Paffrath T, Simanski C, Neugebauer E, Bouillon B. AG Polytrauma of the German Trauma Society (DGU). Early coagulopathy in multiple injury: an analysis from the German Trauma Registry on 8724 patients. *Injury*. 2007; 38(3):298–304. [PubMed: 17214989]
7. Gonzalez E, Moore EE, Moore HB, Chapman MP, Silliman CC, Banerjee A. Trauma-Induced coagulopathy: An institution's 35 year perspective on practice and research. *Scand J Surg*. 2014; 103(2):89–103. [PubMed: 24786172]
8. Wohlaer MV, Moore EE, Thomas S, Sauaia A, Evans E, Harr J, Silliman CC, Ploplis V, Castellino FJ, Walsh M. Early platelet dysfunction: an unrecognized role in the acute coagulopathy of trauma. *J Am Coll Surg*. 2012; 214(5):739–746. [PubMed: 22520693]
9. Kutcher ME, Redick BJ, McCreery RC, Crane IM, Greenberg MD, Cachola LM, Nelson MF, Cohen MJ. Characterization of platelet dysfunction after trauma. *J Trauma Acute Care Surg*. 2012; 73(1):13–19. [PubMed: 22743367]
10. Moore HB, Moore EE, Chapman MP, Gonzalez E, Slaughter AL, Morton AP, D'Alessandro A, Hansen KC, Sauaia A, Banerjee A, et al. Viscoelastic measurements of platelet function, not fibrinogen function, predicts sensitivity to tissue-type plasminogen activator in trauma patients. *J Thromb Haemost*. 2015; 13(10):1878–1887. [PubMed: 26256459]
11. Hoffman M, Monroe DM 3rd. A cell-based model of hemostasis. *Thromb Haemost*. 2001; 85(6):958–965. [PubMed: 11434702]
12. Cardenas JC, Wade CE, Holcomb JB. Mechanisms of trauma-induced coagulopathy. *Curr Opin Hematol*. 2014; 21(5):404–409. [PubMed: 25010798]
13. Fülöp A, Turóczi Z, Garbaisz D, Harsányi L, Szijártó A. Experimental models of hemorrhagic shock: a review. *Eur Surg Res*. 2013; 50(2):57–70. [PubMed: 23615606]
14. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, McDonald-Smith GP, Gao H, Hennessy L, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A*. 2013; 110(9):3507–3512. [PubMed: 23401516]
15. Haworth JS, Elster EA, Fryer D, Sheppard F, Morthole V, Krishnamurthy G, Tomori T, Brown TS, Tadaki DK. Evaluation of lyophilized platelets as an infusible hemostatic agent in experimental non-compressible hemorrhage in swine. *J Thromb Haemost*. 2009; 7(10):1663–1671. [PubMed: 19656278]
16. Griggs TR, Potter JS, McClanahan SB, Webster WP, Brinkhous KM. Macromolecular factor VIII complex: functional and structural heterogeneity observed in von Willebrand swine with transfusion. *Proc Natl Acad Sci U S A*. 1977; 74(2):759–763. [PubMed: 300481]
17. Zhang B, Zhang A, Zhao Y. Platelet aggregation and thrombosis in xenotransplantation between pigs and humans. *Thromb Res*. 2008; 121(4):433–441. [PubMed: 17644165]
18. Hauser CJ. Preclinical models of traumatic, hemorrhagic shock. *Shock*. 2005; 24(Suppl 1):24–32. [PubMed: 16374369]
19. Redl H, Bahrami S. Large animal models: baboons for trauma, shock, and sepsis studies. *Shock*. 2005; 24(Suppl 1):88–93. [PubMed: 16374379]

20. Hoyt DB, Dutton RP, Hauser CJ, Hess JR, Holcomb JB, Kluger Y, Mackway-Jones K, Parr MJ, Rizoli SB, Yukioka T, et al. Management of coagulopathy in the patients with multiple injuries: results from an international survey of clinical practice. *J Trauma*. 2008; 65(4):755–764. discussion 64–5. [PubMed: 18849787]
21. Solomon C, Traintinger S, Ziegler B, Hanke A, Rahe-Meyer N, Voelckel W, Schöchl H. Platelet function following trauma. A multiple electrode aggregometry study. *Thromb Haemost*. 2011; 106(2):322–330. [PubMed: 21655681]
22. Hanke AA, Roberg K, Monaca E, Sellmann T, Weber CF, Rahe-Meyer N, Gorlinger K. Impact of platelet count on results obtained from multiple electrode platelet aggregometry (Multiplate). *Eur J Med Res*. 2010; 15(5):214–219. [PubMed: 20562061]
23. Stissing T, Dridi NP, Ostrowski SR, Bochsén L, Johansson PI. The influence of low platelet count on whole blood aggregometry assessed by Multiplate. *Clin Appl Thromb Hemost*. 2011; 17(6):E211–E217. [PubMed: 21406416]
24. Brass LF, Tomaiuolo M, Stalker TJ. Harnessing the platelet signaling network to produce an optimal hemostatic response. *Hematol Oncol Clin North Am*. 2013; 27(3):381–409. [PubMed: 23714305]
25. D'Alessandro A, Moore HB, Moore EE, Wither M, Nemkov T, Gonzalez E, Slaughter A, Fragoso M, Hansen KC, Silliman CC, et al. Early hemorrhage triggers metabolic responses that build up during prolonged shock. *Am J Physiol Regul Integr Comp Physiol*. 2015; 308(12):R1034–R1044. [PubMed: 25876652]
26. Daley MJ, Enright Z, Nguyen J, Ali S, Clark A, Aydelotte JD, Teixeira PG, Coopwood TB, Brown CV. Adenosine diphosphate platelet dysfunction on thromboelastogram is independently associated with increased mortality in traumatic brain injury. *Eur J Trauma Emerg Surg*. 2016; doi: 10.1007/s00068-016-0643-z
27. Castellino FJ, Chapman MP, Donahue DL, Thomas S, Moore EE, Wohlaue MV, Fritz B, Yount R, Ploplis V, Davis P, et al. Traumatic brain injury causes platelet adenosine diphosphate and arachidonic acid receptor inhibition independent of hemorrhagic shock in humans and rats. *J Trauma Acute Care Surg*. 2014; 76(5):1169–1176. [PubMed: 24747445]
28. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem*. 1998; 273(4):2024–2029. [PubMed: 9442039]
29. Roberts DE, McNicol A, Bose R. Mechanism of collagen activation in human platelets. *J Biol Chem*. 2004; 279(19):19421–19430. [PubMed: 14981087]
30. Murray R, Shipp E, FitzGerald GA. Prostaglandin endoperoxide/thromboxane A2 receptor desensitization. Cross-talk with adenylate cyclase in human platelets. *J Biol Chem*. 1990; 265(35):21670–21675. [PubMed: 1701435]
31. Okwu AK, Ullian ME, Halushka PV. Homologous desensitization of human platelet thromboxane A2/prostaglandin H2 receptors. *J Pharmacol Exp Ther*. 1992; 262(1):238–245. [PubMed: 1378092]
32. Gryglewski RJ. Prostacyclin among prostanoids. *Pharmacol Rep*. 2008; 60(1):3–11. [PubMed: 18276980]
33. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol*. 2010; 30(12):2341–2349. [PubMed: 21071698]
34. Letson HL, Dobson GP. Differential contributions of platelets and fibrinogen to early coagulopathy in a rat model of hemorrhagic shock. *Thromb Res*. 2016; 141:58–65. [PubMed: 26970714]
35. Sirajuddin S, Valdez C, DePalma L, Maluso P, Singhal R, Schroeder M, Sarani B. Inhibition of platelet function is common following even minor injury. *J Trauma Acute Care Surg*. 2016; 81:328–332. [PubMed: 27027558]
36. Moore HB, Moore EE, Morton AP, Gonzalez E, Fragoso M, Chapman MP, Dzieciatkowska M, Hansen KC, Banerjee A, Sauaia A, et al. Shock-induced systemic hyperfibrinolysis is attenuated by plasma-first resuscitation. *J Trauma Acute Care Surg*. 2015; 79(6):897–903. discussion 903–904. [PubMed: 26680132]

Figure 1.

Procedure summary. Schematic depicting experimental design including time points of injury window, hemorrhage, resuscitation, and observational procedures. Whole blood was drawn for laboratory tests (complete blood cell count, arterial blood gas, and platelet impedance aggregometry analysis) at BSLN, EOS ($T = 60$ minutes), EOR ($T = 180$ minutes), and $T = 360$ minutes.

Figure 2.

Platelet count. Quantified circulating platelet count from complete blood cell count at BSLN, EOS ($T = 60$ minutes), EOR ($T = 180$ minutes), and $T = 360$ minutes for combined cohorts. Shaded area reflects normal platelet count range ($193\text{--}414 \times 10^3/\mu\text{L}$) for rhesus macaque determined in house. Limit for thrombocytopenia ($<100 \times 10^3/\mu\text{L}$) is depicted by (•••). Paired t -test statistical analysis was performed with $p < 0.05$ considered significant ($p < 0.001$ *** and $p < 0.0001$ ****); data shown as mean $\pm$ SD.

Figure 3.

Platelet impedance aggregometry. Multiplate platelet aggregation was quantified using the area under the curve in units (U) produced by platelet agonists at BSLN, EOS ($T = 60$ minutes), EOR ($T = 180$ minutes), and $T = 360$ minutes in NHP whole blood. Platelets function in response to (A) ADP, (B) COL, (C) TRAP, and (D) AA. Wilcoxon nonparametric test statistical analysis was performed with $p < 0.05$ considered significant ($p < 0.001$ *** and $p < 0.0001$ ****); data shown as median and IQR.

Figure 4.

Spearman correlations of platelet count and aggregation. Scatter plots showing correlation between platelet count and (A) ADP, (B) COL, (C) TRAP, and (D) AA responsiveness using matched data from all time points collected. Platelet aggregation was quantified using area under the curve in units (U). Rho values for each pairwise correlation given at top left of each graph, with all associated $p < 0.0001$.

Figure 5.

Platelet impedance aggregometry (normalized to platelet count). Multiplate platelet aggregation was quantified using the area under the curve in units (U) produced by platelet agonists normalized to platelet count at BSLN, EOS ($T = 60$ minutes), EOR ($T = 180$ minutes), and $T = 360$ minutes in NHP whole blood. Platelets function in response to (A) ADP, (B) COL, (C) TRAP, and (D) AA. Wilcoxon nonparametric test statistical analysis was performed with $p < 0.05$ considered significant ($p < 0.001$ *** and $p < 0.0001$ ****); data shown as median and IQR.

Clinical Markers of Shock

TABLE 1

Parameter	Cohort	Injury Pattern	BSLN	EOS	EOR	T360
Lactate, mmol/L	HS	HS	1.2 ± 0.4	2.8 ± 1.3	0.8 ± 0.2	0.9 ± 0.3
		HS-ST	1.4 ± 0.2	3.2 ± 1.7	1.0 ± 0.2	1.0 ± 0.2
		HS-ST-MS	1.4 ± 0.3	3.3 ± 2.2	1.2 ± 0.4	1.4 ± 0.6
Base deficit, mmol/L	HS	HS	0.0 ± 0.0	-4.9 ± 2.8	-7.8 ± 2.4	-7.6 ± 2.2
		HS-ST	0.0 ± 0.0	-4.1 ± 3.3	-7.3 ± 1.3	-6.8 ± 1.1
		HS-ST-MS	0.0 ± 0.0	-4.8 ± 3.4	-7.5 ± 1.2	-6.7 ± 1.8
pH	HS	HS	7.45 ± 0.02	7.46 ± 0.06	7.38 ± 0.03	7.39 ± 0.03
		HS-ST	7.45 ± 0.03	7.46 ± 0.07	7.38 ± 0.03	7.39 ± 0.02
		HS-ST-MS	7.44 ± 0.04	7.45 ± 0.05	7.39 ± 0.04	7.40 ± 0.04
HCO ₃ ⁻ , mmol/L	HS	HS	30.6 ± 2.4	24.6 ± 3.0	23.1 ± 1.5	23.1 ± 1.4
		HS-ST	30.8 ± 0.8	25.8 ± 3.3	23.9 ± 1.2	24.2 ± 0.7
		HS-ST-MS	31.1 ± 1.8	25.1 ± 4.2	23.4 ± 1.1	23.9 ± 0.7
pCO ₂ , mm Hg	HS	HS	43.8 ± 3.9	34.4 ± 4.5	39.1 ± 3.7	37.9 ± 2.0
		HS-ST	44.3 ± 2.6	36.4 ± 4.5	40.4 ± 3.1	39.8 ± 2.8
		HS-ST-MS	46.5 ± 5.5	36.3 ± 8.0	39.3 ± 4.5	38.5 ± 4.3
Platelet count, 10 ³ /μL	HS	HS	296 ± 54	224 ± 67	210 ± 40	215 ± 74
		HS-ST	283 ± 45	246 ± 62	199 ± 39	218 ± 34
		HS-ST-MS	264 ± 25	238 ± 36	192 ± 29	200 ± 26

Values for blood lactate, base deficit, pH, HCO₃⁻, pCO₂, and platelet count for HS, HS-ST, and HS-ST-MS cohorts at BSLN, EOS ($T = 60$ minutes), EOR ($T = 180$ minutes), and $T = 360$ minutes. Two-way repeated-measures analysis of variance statistical analyses were performed with $p < 0.05$ considered significant; data shown as mean ± SD.