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Activated Platelets in Heparinized Shed Blood: The "Second-Hit" of Acute Lung Injury in Trauma/Hemorrhagic Shock Models

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Abstract

Background—The return of heparinized shed blood in trauma/hemorrhagic shock (T/HS) models remains controversial due to potential anti-inflammatory properties. Although ubiquitous as an anticoagulant, heparin is ineffective on cellular coagulation as an antithrombotic agent. Therefore, we hypothesized that returning heparinized shed blood would paradoxically enhance ALI following T/HS due to the infusion of activated platelets.

Methods—Sprague-Dawley rats, anesthetized with pentobarbital, underwent laparotomy and hemorrhage-induced shock (MAP of 30 mmHg × 45 min). Animals were resuscitated with a combination of normal saline (NS) and returned shed blood. Shed blood was collected in either 80U/kg of heparin, 800U/kgof heparin, citrate, or diluted 1:8 with NS. An additional group of animals were pretreated with a platelet $P2Y_{12}$ receptor antagonist (clopidogrel) prior to T/HS. BAL, lung MPO assays, pulmonary immunofluorescence, and blood smears were conducted.

Results—BAL protein increased in animals resuscitated with heparinized shed blood ($p<0.0001$). Blood smears and platelet function assays revealed platelet aggregates and increased platelet activation. Animals pretreated with a platelet $P2Y_{12}$ receptor antagonist were protected from post-injury ALI ($p<0.0001$). Animals with return of shed blood had increased pulmonary PMN sequestration ($p<0.0001$). Pulmonary immunofluorescence demonstrated microthrombi only in the T/HS group receiving heparinized shed blood ($p<0.0001$).

Conclusion—The return of heparinized shed blood functions as a "second-hit" to enhance ALI, with activated platelets propagating microthrombi and pulmonary PMN recruitment.

Keywords

Heparin; platelets; microthrombi; trauma/hemorrhagic shock; acute lung injury

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Introduction

Clinically relevant studies on the pathophysiology of trauma and hypovolemic shock include a resuscitation phase using blood products, often carried out by the return of shed blood. However, returning shed blood requires the addition of an anticoagulant, which may possess confounding properties. Consequently, the use of heparin and the return of heparinized shed blood in animal trauma/hemorrhagic shock (T/HS) models have raised considerable controversy. Heparin has been the anticoagulant of choice because it is inexpensive and the mechanism does not involve chelation of calcium, which is important for many physiological processes. Since the discovery of heparin in 1916, its mechanism of action, therapeutic effects, and clinical risks have been debated.[1] Although widely accepted clinically as an effective anticoagulant, heparin does not possess direct anticoagulant properties, but rather inhibits the fluid phase of coagulation indirectly through activation of antithrombin III. Antithrombin III binds to and inactivates thrombin, forming a thrombin-antithrombin (TAT) complex, and inhibits factor Xa. In the presence of heparin, this reaction is accelerated approximately 1000-fold.[2] The additional concentration-dependent physiologic effects of heparin include anti-inflammatory properties, alteration of platelet aggregation, increased electronegative potential of the endothelium, activation of lipoprotein lipases, and suppression of aldosterone secretion.[1] These confounding effects of heparin have raised the question of its relevance in animal models, specifically those involving trauma and inflammation.

However, designing clinically relevant T/HS animal models has been challenging with the removal of heparinized shed blood from resuscitation. Historically, heparin was needed to maintain the patency of arterial and venous catheters, but modifications in techniques have been able to eliminate the use of heparin for this purpose. Currently, the predominant use of heparin is to anticoagulate shed blood for its return during resuscitation. Alternatively, eliminating the return of shed blood complicates these models via reduced oncotic pressure, and the development of critical anemia, especially at higher altitudes with significantly decreased partial pressures of oxygen. [3]

In spite of these long-standing controversies, there is a paucity of research evaluating the direct effects of heparin in T/HS models and subsequent acute lung injury(ALI). These are timely questions, since heparin has now been implicated in generating toxic factors in mesenteric lymph,[4] a known conduit for mediators augmenting ALI in animal models.[5] Therefore, the purpose of our study was to determine the effects of heparin and the return of heparinized shed blood in a clinically relevant animal model of T/HS mediated ALI.

Methods

Adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 350–425 g were housed under barrier-sustained conditions with 12 hr light/dark cycles and allowed free access to food and water for a minimum of one week before use. All animals were maintained in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, and this study was approved by the University of Colorado Denver Animal Care and Use Committee.

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Platelet function assay equipment and supplies were provided by Haemonetics Corporation (Niles, IL); 0.9% injection grade normal saline (NS) was purchased from Baxter Healthcare (Deerfield, IL); Sodium pentobarbital was purchased from Abbott Labs (Chicago, IL); Polyethylene tubing was acquired from Fisher Scientific (Pittsburgh, PA).

Continuous blood pressure measurement was performed using a ProPaq invasive monitoring device (Welch-Allyn, Skaneateles Falls, NY).

Trauma/Hemorrhagic Shock Model

Animals (n=26) were anesthetized with 50 mg/kg sodium pentobarbital, and were given a subcutaneous injection of 1% lidocaine for local anesthesia. The femoral artery and vein were cannulated with polyethylene (PE-50) tubing for continuous invasive pressure monitoring and to establish venous access. A tracheotomy was performed, at which point the animal was placed on 30% FiO_2 using an air-oxygen mixer (Sechrist, Anaheim, CA) at a flow rate of 2 liters/minute. The animal's body temperature was measured rectally and kept euthermic with the use of a heat lamp. After a 45-minute observation period, a laparotomy was performed to simulate trauma, and closed in a two-layer fashion using a 3-0 non-absorbable monofilament suture. Controlled hemorrhage was then induced over a period of 10-minutes through the arterial catheter to maintain a MAP of 30 mmHg for 45 minutes. To distinguish the effects of heparin, shed blood was collected either with 80 Units/kg of heparin, 800 Units/kg of heparin, citrate, or diluted 1:8 with NS without the use of heparin, and stored at 37°C. At the end of shock, to simulate clinical practice, animals were resuscitated with twice their shed blood (SB) volume in NS over a 30-minute period, followed by ½ of the SB volume over 30 minutes, and an additional hour of resuscitation with twice the SB volume in NS. The animals were observed for another hour prior to being euthanized with a pentobarbital overdose. The T/HS cohort who did not receive shed blood, were given four times their SB volume in NS over a 2-hour period.

Sham Animals

Trauma/Sham Shock (T/SS) animals (n=5) and heparin T/SS animals (n=4) were anesthetized with 50 mg/kg sodium pentobarbital, and were given a subcutaneous injection of 1% lidocaine for local anesthesia. The femoral artery and vein were then cannulated. A tracheotomy was performed, and the animal was placed on 30% FiO_2 using an air-oxygen mixer at a flow rate of 2 liters/minute. The animal was kept euthermic with the use of a heat lamp. After a 45-minute observation period, a laparotomy was performed to simulate trauma. No hemorrhagic shock was performed and T/SS animals were observed for 3 hours prior to being euthanized with a pentobarbital overdose. The heparin T/SS animals were given 80 Units/kg of heparin IV 30 minutes following the initial 45 minute observation period.(the same time period in which the shed blood was returned in the T/HS arm).

Exchange Transfusion

Animals (n=3) were subjected to identical T/SS as reported above. However, during the same period of shed blood return in the T/HS model, animals were infused heparinized shed blood (5 ml/30 min) from an animal that just recently underwent T/HS. For each 0.5 ml of shed blood transfused, 0.5 ml of the T/SS animal's native blood was removed to maintain euvolemia. Animals were observed for 3 hours following shock, and were euthanized with a pentobarbital overdose.

Platelet Function Assay

Thrombelestography (TEG) has effectively been used in rodent models to evaluate the individual components of coagulation.[6] Therefore, platelet function was determined by utilizing a TEG-derived platelet function assay -PlateletMapping™.[7] Briefly, a baseline 900μl blood sample was collected in a vial containing 100 μl of 0.3% heparin or 100 μl of 4% citrate. Shed blood during shock was collected in a similar manner. The blood sample was inverted 5 times and was set on its side, undisturbed, for 30 minutes. Next, 360 μl of the blood sample was placed into the pre-warmed cup of the TEG analyzer, and 10μl of the

prepared activator (Reptilase, Factor XIII, and phospholipids) was added. Reptilase cleaves fibrinogen to fibrin in the absence of thrombin, and eliminates thrombin-mediated activation of platelets. Factor XIII and phospholipids allow for the cross-linking of fibrin and clot formation in a thrombin-independent fashion only if the platelet GPIIb/IIIa receptors are activated. Since platelet activation was being measured instead of inhibition, there was no addition of arachidonic acid (AA) or ADP as performed in standard PlateletMapping™. An increase in the tracing's amplitude thus reflects GPIIb/IIIa receptor activation.

Platelet Inhibition

Animals (n=5) were pre-treated with a 20 mg/kg oral dose of a $P2Y_{12}$ receptor antagonist (clopidogrel) (Sanofi-Aventis Pharmaceuticals, Bridgewater, NJ) daily for 3 consecutive days prior to undergoing T/HS. Animals were then subjected to the identical T/HS described above with the shed blood collected on 80 Units/kg of heparin.

Lung Neutrophil Accumulation

At the end of the experiment, the right lung was procured, frozen, and stored at −80°C. On the day of the assay, the lung specimen was thawed, weighed, and homogenized in 10 mL of 20 mM potassium phosphate buffer (PPB) and centrifuged at 40,000g for 30 minutes. The pellet was sonicated for 90 seconds in 4 mL of 50 mM PPB containing 5 g/L hexadecyltrimethyl ammonium bromide, incubated at 60°C for 2 hours, and then centrifuged at 5,000g for 10 minutes. The supernatant (5 μL) was added to a 96-well plate, and 145 μL of 50 mM PPB containing 0.167 mg/mL O-dianisidine and 0.5% hydrogen peroxide was added to each well. The absorbance at 460 nm was measured with a read time of 5 min at 10-second intervals using a spectrophotometer (Molecular Devices Corp, Sunnyvale, CA) to determine myeloperoxidase (MPO) activity.

Lung Vascular Permeability

Following the pentobarbital overdose, a BAL was performed through the tracheotomy tube. Normal saline (5 ml)was injected into the trachea, aspirated, and collected. This procedure was repeated three times. The return of bronchoalveolar lavage fluid (BALF) was consistently greater than 12 mL. The BAL fluid was frozen, and stored at −80°C until the assay was performed. The Pierce BCA protein assay kit (Thermo Scientific) was used to measure BAL protein, reflecting lung vascular permeability. A standard curve was generated by adding specific concentrations of albumin to 8 wells in a 96-wellplate. Next, 12.5 μl of BAL fluid were added to empty wells in duplicate for each experiment, followed by 12.5 μl of distilled H_2O. Subsequently, 250 μl of the working reagent was added to each well, and the plate incubated at 37°C for 30 min. The absorbance at 562 nm was then measured using a spectrophotometer (Molecular Devices Corp, Sunnyvale, CA) to determine the protein concentration.

Immunofluorescence

At the end of the experiment, the left lung was inflated with 4% paraformaldehyde in PBS and fixed overnight. The lung was then inflated with 1.5 ml OCT in 4% sucrose, sectioned, and embedded in OCT prior to freezing. Frozen sections (5 μm) were cut, and placed on glass slides. The sections were then washed in phosphate-buffered saline (PBS)pH 7.4, and permeabilized with a 70% acetone/30% methanol solution. The slides were then treated with a 1:400 dilution of Re-Blot™ (Millipore, Billerica, MA) in distilled H_2O, followed by three washes in PBS. A10% normal donkey serum in PBS blocking solution was applied to the sections for 1 hour. The primary antibodies, as well as isotype controls, were applied and incubated overnight at 4°C. The sections were again washed in PBS, and anti-species fluorescent antibodies were applied for 1 hour in a dark room. Three final washings with

PBS occurred, and Prolong Plus solution containing DAPI was added prior to the placing of a cover slip.

Fibrin was labeled with a sheep polyclonal anti-fibrinogen antibody (Serotec, Raleigh, NC). Platelets were labeled with a mouse polyclonal anti-integrin αIIb (CD41) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and neutrophils were labeled with rabbit polyclonal anti-PMN serum (Accurate Chemical, Westbury, NY). As a secondary label for the fibrinogen, a 488-nm (green) labeled donkey anti-sheep IgG was used. A 647-nm (far red) labeled donkey anti-rabbit IgG was used to label neutrophils, and a Cy3 (red) donkey anti-mouse IgG was used to label platelets. Normal sheep IgG(Jackson Immuno Research, West Grove, PA), normal mouse IgG (Jackson Immuno Research), and normal rabbit serum (Jackson Immuno Research) were used as isotype controls.

Peripheral Blood Smears

Peripheral blood smears were made by placing one drop of shed blood on a glass slide, and performing the smear by the standard method. The slide was then air-dried, and sent to the Hematopathology department at Children's Hospital Denver for modified Wrights' staining. The slides were evaluated by a hematopathologist to determine platelet morphology and aggregation.

Statistical Analysis

BAL protein, MPO, and immunofluorescence data are reported as the mean ± standard deviation and were compared by analysis of variance using the Bonferroni/Dunn test for post hoc comparisons. The results of the PlateletMapping™ assay are reported as the mean ± standard deviation and were compared by a paired student's t-test. A p-value <0.001 was considered statistically significant.

Results

Heparin Effects in the T/HS Model

Animals were subjected to trauma/sham shock (T/SS), trauma/hemorrhagic shock with no heparin and no shed blood returned (T/HS no SB), or a T/HS arm in which heparinized shed blood was returned during resuscitation (T/HS + 80U/kg Hep). ALI, as measured by BALF protein, revealed that animals who underwent T/HS without the return of shed blood, developed significant lung injury (1.13 ± 0.07mg/ml) compared to T/SS (0.52 ± 0.17 mg/ml) (Figure 1). Animals subjected to T/HS with the return of heparinized shed blood had an exacerbation of lung injury (1.62 ± 0.29).

To determine if heparin was an independent factor, T/SS animals were given the same dose of heparin during the time period in which blood would have been transfused (Heparin T/SS), and there was no evidence of lung injury compared to the T/SS group (0.51 ± 0.16 vs. 0.52 ± 0.17 mg/ml). In addition, a group of animals undergoing T/HS had their shed blood collected in 10 times the amount of heparin (T/HS + 800U/kg Hep), which was returned during resuscitation. These animals had a mild decrease in lung injury compared to the T/HS + 80U/kg Hep group, which was not statistically significant (1.30 ± 0.15 vs. 1.62 ± 0.29mg/ml)(Figure 1).

Anticoagulant Effect of Heparin

To examine if the anticoagulant properties of heparin contributed to ALI, shed blood was collected in a calcium-chelating agent (citrate) or diluted 1:8 in NS to eliminate the confounding properties of heparin. Both the citrate and blood dilution groups had similar

levels of lung injury (0.70 ± 0.09 and 0.66 ± 0.18 mg/ml respectively); levels modestly higher compared to the T/SS group, but were not statistically significant.

Heparin Effect on Platelets

Since heparin by itself does not provoke ALI, and other methods of anticoagulating shed blood (calcium chelation and factor dilution) attenuate ALI, the anticoagulant properties of heparin were suspect. Peripheral blood smears were obtained from samples of shed blood that were collected in 80 Units/kg of heparin, 800 Units/kg of heparin, citrate, or diluted 1:8 with NS. A hematopathologist evaluated the slides and found numerous large platelet aggregates in the shed blood collected in 80 Units/kg of heparin, a few small platelet aggregates in the shed blood collected in 800 Units/kg of heparin, rare, very small platelet aggregates in the shed blood collected in citrate, and rare platelets and no aggregates in the blood dilution group (Figure 2).

Knowing that heparin primarily alters the fluid phase of coagulation through antithrombin III, its effect on the cellular components of shed blood (platelets) was further interrogated. Modified PlateletMapping™ was employed to determine the platelet function in shed blood collected in heparin or in citrate. At baseline, both samples showed normal tracings with no platelet activation (80 Units/kg heparin G-value 0.02 ± 0.45 vs. citrate G-value 0.06 ± 0.06). However, platelet mapping performed on shed blood at the end of shock revealed increased platelet activation in the heparinized shed blood group (80 Units/kg heparin G-value 2.23 ± 0.84 vs. citrate G-value 0.1 ± 0.00) ($p < 0.001$). There was no change in the citrate group from baseline to shock, confirming that in the presence of chelated calcium, the platelet-fibrin interaction is inhibited(Figure 3).

Platelet Inhibition/Mechanism

Platelet-PMN interactions are important in the pathogenesis of ALI, and in part, mediated through the platelet ADP ($P2Y_{12}$) receptor.[8] Having shown that heparinized shed blood contained platelet aggregates and activated platelets, animals were given a platelet $P2Y_{12}$ receptor antagonist (clopidogrel). Animals pre-treated with a $P2Y_{12}$ receptor antagonist had lung injury comparable to sham animals despite the return of heparinized shed blood (0.57 + 0.27 vs. 0.52 ± 0.17mg/ml)(Figure 4).

To determine if activated platelets/platelet aggregates in shed blood were sufficient for ALI, shed blood collected in 80 Units/kg of heparin was transfused into a T/SS animal. These shed blood recipients had no evidence of ALI, and BALF protein levels were similar to T/SS animals (0.48 ± 0.03 vs. 0.52 ± 0.17mg/ml)(Figure 4).

Two-Hit Mechanism

Recognizing that activated platelets/platelet aggregates were necessary, but not sufficient for ALI, the effects of heparinin T/HS and inflammation were evaluated. Since pulmonary endothelial activation and pulmonary sequestration of neutrophils are a crucial component of ALI,[9] an MPO assay on homogenized lung tissue was performed. Animals who did not undergo hemorrhagic shock and who did not receive shed blood had comparable MPO levels to the T/SS group (heparin T/SS 3.55 ± 0.69, exchange transfusion 3.95 ± 1.15 vs. T/SS 3.58 ± 1.06). Animals who underwent T/HS without the return of shed blood had significantly increased MPO levels (T/HS no SB8.83 ±0.54). All T/HS groups who received shed blood, except the T/HS clopidogrel + heparin group, had significantly increased MPO activity compared to the T/SS group(T/HS + 80U/kg hep 5.52 ± 0.31, T/HS ± 800U/kg hep 5.67 ± 0.78, T/HS citrate 5.74 ± 0.84, T/HS blood dilution 6.68 ± 0.53 vs. T/HS clopidogrel + hep 4.33 ± 0.58) (Figure 5). Groups who underwent T/HS and received shed blood with activated platelets/platelet aggregates developed lung injury. Therefore, suggesting that both

sequestered pulmonary neutrophils and activated platelets are involved in the genesis of post-shock ALI.

Pulmonary Microthrombi

Microthrombi have been implicated in the development of ALI.[10,11] The principal composition of these thrombi include fibrinogen, platelets, and neutrophils.[12] These components were examined by immunofluorescence in pulmonary histological sections (Figure 6). The mean sum intensity of fluorescently labeled fibrinogen, platelets, and neutrophils where measured for each group (Figure 7). The T/HS group with the returned blood collected on 80 Units/kg of heparin, had the highest immunofluorescence of fibrinogen, platelets, and neutrophils for all groups, but were only significant for fibrinogen and neutrophils ($p<0.0001$). Furthermore, significant colocalization of fibrinogen, platelets, and neutrophils were only observed in the T/HS group($p<0.0001$).

Discussion

These data show that the return of heparinized shed blood in animal T/HS models exacerbates ALI indirectly through the return of activated platelets and not the direct effects of heparin itself. As a molecule, heparin is evolutionarily conserved, and present in invertebrate species lacking a coagulation system, suggesting other primary roles, such as host defense, rather than anticoagulation.[13,14] Though produced by endothelial cells, heparins are highly negatively charged glycans that have many other ionic interactions responsible for its mechanistic diversity. In spite of this fact, heparin has been widely accepted clinically as an effective anticoagulant, but few have questioned the multiple physiological effects of this molecule. A review of the literature favors heparin as protective in T/HS models,[15,16] but heparin has also been implicated as an injurious molecule,[4] further adding to the controversy[17] of heparin use in T/HS models. Those involved in basic science research have therefore debated the use of heparin in animal models for these reasons. Yet, animal models utilizing the return of heparinized shed blood continue to be exploited.

Although a model not used in this study, we have employed heparin for the return of shed blood in our T/HS lung injury model with mesenteric lymph diversion[18], because the return of shed blood is critical for maintaining both oncotic pressure, due to the excessive protein loss in lymph diversion, and appropriate hemoglobin levels to maintain sufficient oxygen carrying capcity.[19] Therefore, a study to determine the effects of heparin in these clinically relevant animal models of T/HS mediate lung injury was warranted. Initially, we found that heparin infusion in the setting of sham shock did not provoke ALI, but in the presence of shock and the return of shed blood, heparin exacerbated lung injury implicating the heparinized shed blood. Thus, shed blood was collected in 10 times the dose of heparin (800 Units/kg) to ensure adequate thrombin inhibition, but animals still developed ALI. Other anticoagulation methods of shed blood were employed, such as calcium chelation and factor dilution, and both were protective against ALI implicating a pathological process with heparin use in shed blood storage. Those involved with blood banking have abandoned the use of heparin for whole-blood storage due to increased platelet loss and hemolysis.[20] The platelet loss was most likely secondary to the formation of platelet aggregates as observed in heparinized animal models in which “white” thrombi developed extracorporeally.[21] Therefore, the shed blood was examined by obtaining peripheral smears, and demonstrated that although heparin was effective in inhibiting the fluid phase of coagulation, it was a poor antithrombotic agent, unable to prevent platelet activation/aggregation. This was confirmed by platelet function assays.

At baseline, PlateletMapping™ confirmed that 80 Units/kg of heparin was sufficient to prevent thrombin-mediated activation of platelets. However, shed blood collected during T/

HS resulted in GPIIb/IIIa activation in spite of thrombin inhibition. Therefore, other endogenous mediators of T/HS must be involved in the activation of platelets. In-vivo platelet activation can occur via multiple platelet agonist/receptor interactions during T/HS; collagen (GP IV), thrombin (PAR1 and PAR4), ADP ($P2Y_1$ and $P2Y_{12}$), thromboxane A_2 (TP), and epinephrine (α_{2A}).[22] Furthermore, when blood is removed and stored ex-vivo during T/HS, the endothelial-derived inhibitors of platelet activation (NO, PGI_2, and CD39-ADPase) are removed, further exacerbating platelet activation. Consequently, animals pretreated with a platelet $P2Y_{12}$ receptor antagonist (clopidogrel), had a protective effect in spite of T/HS and the return of heparinized shed blood. Thus, implicating activated platelets in the pathogenesis of ALI and that heparin use for the storage of shed blood promotes platelet activation ex-vivo.

However, T/HS without the return of shed blood is sufficient to provoke ALI. Therefore, platelet activation must occur in-vivo during T/HS through potentially several different receptors. Growing evidence is showing that the platelet-endothelium interaction mediated through platelet P-selectin is vital in the pathogenesis of acute lung injury.[23] Consequently, when heparinized shed blood is returned, containing an abundant source of activated platelets, the pathogenesis of ALI may be accelerated.

Calcium chelation and factor dilution appeared protective compared to the T/HS group who did not receive shed blood. Since coagulation and thrombosis are both dependent on extracellular calcium, as well as cytosolic calcium for platelet intracellular signaling, calcium chelation and dilution prevented platelet activation as evidenced by the platelet function assay. However, the protective effects of the T/HS citrate and T/HS blood dilution groups compared to the T/HS no SB group, are likely due to the return of shed blood (not containing activated platelets), ultimately improving their resuscitation through increased oxygen carrying capacity and oncotic pressures. As the T/HS + 800 Units/kg heparin group did not show any statistically significant decrease from the T/HS + 80 Units/kg heparin group, the citrate and blood dilution groups had no statistically change compared to the T/SS group. It is important to note that this study is likely underpowered to detect significant changes between these groups; however, these groups provided the necessary information to implicate platelets in ALI.

To determine the effects of activated platelets in the lung, histological sections were evaluated for microthrombi and were fluorescently labeled with antibodies against platelets, fibrinogen, and neutrophils. Animals undergoing T/HS with the return of heparinized shed blood had significantly higher levels of fibrinogen and neutrophils. Upon colocalization of all three microthrombi components (fibrinogen, platelets, and neutrophils), microthrombi were only significantly found in the T/HS group who received heparinized shed blood.

Minimal microthrombi were noted in the T/HS no shed blood group, which suggest that without the return of shed blood, only minimal thrombi were generated following NS resuscitation secondary to platelet and factor dilution. This raises the probability of other mechanisms for ALI in models not utilizing the return of shed blood, such as pulmonary ischemia secondary to anemia and decreased oxygen carrying capacity rather than microthrombi. However, microthrombi have been observed clinically in the setting of post-injury ALI/ARDS.[10,24] This implies microthrombi formation may be essential for the development of lung injury, and suggests a critical role for platelets in post-traumatic organ failure. Therefore, a model utilizing the return of shed blood, which produces an outcome observed clinically (pulmonary microthrombi), is consistent and translates to known clinical scenarios.

To further address this question of mechanism, heparinized shed blood was transfused into a T/SS animal (exchange transfusion group), and no lung injury was detected. Therefore, activated platelets in shed blood were not sufficient for ALI, suggesting a two-event mechanism. During the initial insult, the pulmonary endothelium is activated allowing for neutrophil adhesion and emigration. A second insult is then required to activate the neutrophils, which triggers a cytokine release, resulting in endothelial leak and lung injury. Our MPO data show that an initial insult of T/HS is sufficient for pulmonary neutrophil sequestration, but not necessarily lung injury. It is the transfusion of heparinized shed blood which acts as the second-hit through activated platelets, triggering a cytokine release, and generating pulmonary capillary leak.

In antibody-mediated transfusion-related acute lung injury (TRALI), the Matthay group has described a two-event model in which neutrophil depletion, platelet depletion, or treatment with aspirin protected mice from TRALI.[25] Although the mechanism remains unclear, this group suggested pulmonary platelet sequestration was dependent on neutrophils, but platelet depletion did not affect pulmonary neutrophil accumulation. Our study has shown similar results with platelet inhibition utilizing a $P2Y_{12}$ receptor antagonist. However, in spite of a mild decrease in pulmonary neutrophil accumulation in the T/HS clopidogrel + hep group, these results were not significantly different.

Although pulmonary ischemia has some mechanistic merit, it does not fully describe the effects seen in returned shed blood models. However, if shock is prolonged in non-shed blood models, microthrombi may still develop.[11] Thus, platelet activation may occur in-vivo, but at a slower rate than in ex-vivo storage. It is the transfusion of heparinized shed blood, supplying activated platelets, which accelerates the pathogenesis of ALI. Therefore, T/HS is necessary to prime the pulmonary endothelium, and activated platelets act as the “second-hit” enhancing pulmonary PMN sequestration and the formation of pulmonary microthrombi.

Since mesenteric lymph diversion attenuates ALI following T/HS, our laboratory continues to evaluate gut-derived factors in mesenteric lymph for the crucial mediators in the pathogenesis of ALI. Both protein and lipid substrates have been implicated, but have not been fully determined.[18,26] Recently, heparin use, which is known to activate lipoprotein lipase,[27] was found to increase lipase levels in mesenteric lymph following T/HS, which resulted in HUVEC toxicity.[4] Although in-vitro data are suggestive, the clinical relevance remains to be determined. Similarly, as a limitation to all animal research, results of this study may not be extrapolated to the clinical sphere. However, clinical evidence currently supports no untoward effects of heparin use. Cardiac patients undergoing cardiopulmonary bypass procedures (a form of controlled trauma and shock), in spite of high dose heparin use, have a very low incidence of lung injury(0.5–1.0%).[28,29] In contrast, the incidence of lung injury following trauma has been reported as high as 25%.[30] In our recent study evaluating post-traumatic ICU patients with an injury severity score > 15, the incidence of lung dysfunction was greater than 70%.[31] This phenomenon is unexpected since patients undergoing cardiopulmonary bypass (CPB) are older and have higher comorbidities, which are significant risk factors for multiple organ failure.[32,33]

Interestingly, CPB results in a 40–60% reduction in circulating platelets due to contact with the ex-vivo circulatatory system and decreases the sensitivity of the remaining platelets to aggregating agents.[34] These data further implicate the significance of platelets in ALI. In contrast, a recent retrospective study from our institution has associated thrombocytopenia with MOF and worse outcomes.[35] However, the relationship between lung injury and pulmonary platelet sequestration/aggregation in trauma patients, accounting for the thrombocytopenia, has been observed in animal models,[36] as well as clinically.[37]

With the use of any drug in in-vivo models, unintentional effects must be assumed, and controlling for these effects is difficult. Although heparin may possess confounding properties, its use in T/HS models can generate a reproducible and accelerated model of ALI with similar pathological outcomes observed clinically. Investigators must have some understanding of these potential effects in order to properly interpret the results of studies utilizing heparin, but heparin use should not be automatically discounted. The return of heparinized shed blood in T/HS models most accurately simulates clinical resuscitative scenarios, and offers other benefits including maintenance of oncotic pressure and prevention of critical anemia. Furthermore, the return of heparinized shed blood likely enhances the clinical pathogenesis of ALI. It is important to note that heparin use does not directly activate platelets, but it is the failure of heparin to prevent platelet activation ex-vivo, which augments post-injury ALI. In spite of this, there are possible unintended consequences of heparin use, and investigators must determine if the use of heparin will confound outcomes in their own models.

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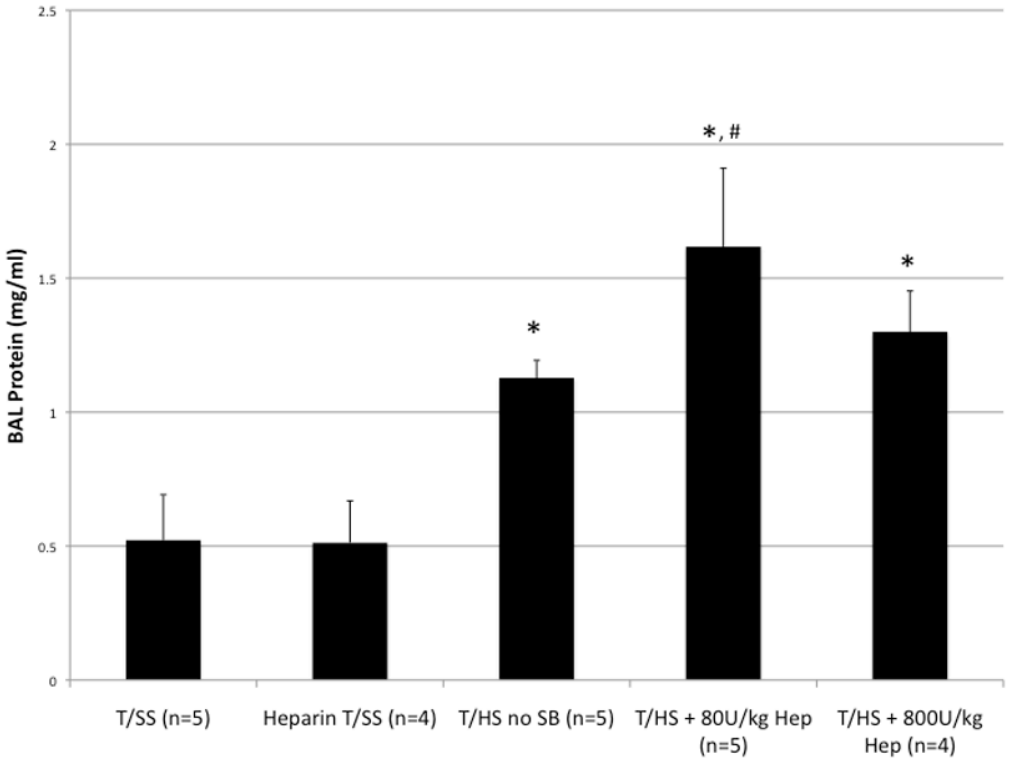

Fig. 1.

Bronchoalveolar lavage protein measured in T/SS, heparin T/SS, T/HS no SB, T/HS + 80 U/kg, and T/HS + 800 U/kg groups. Trauma/hemorrhagic shock without the return of SB is sufficient to incite ALI. However, T/HS with the return of heparinized SB exacerbates ALI. Heparin use in the T/SS group does not affect lung injury. **P*< 0.001 compared with T/SS group. †*P*< 0.001 compared with T/HS–no SB group.

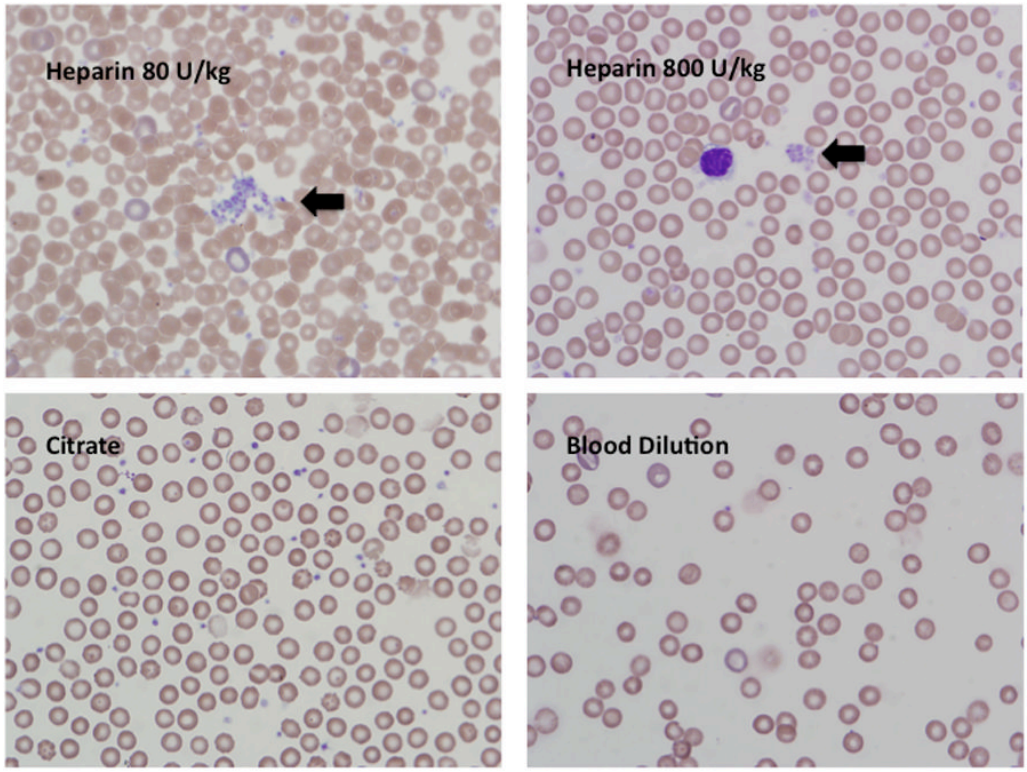

Fig. 2.
Peripheral blood smears of T/HS heparin 80 U/kg, T/HS heparin 800 U/kg, T/HS citrate, and T/HS blood dilution groups. Shed blood collected in both heparin groups contained large platelet aggregates. Shed blood collected in citrate contained small platelet aggregates and SB diluted 1:8 contained rare platelets and no platelet aggregates, indicating that heparin is ineffective in preventing platelet aggregation.

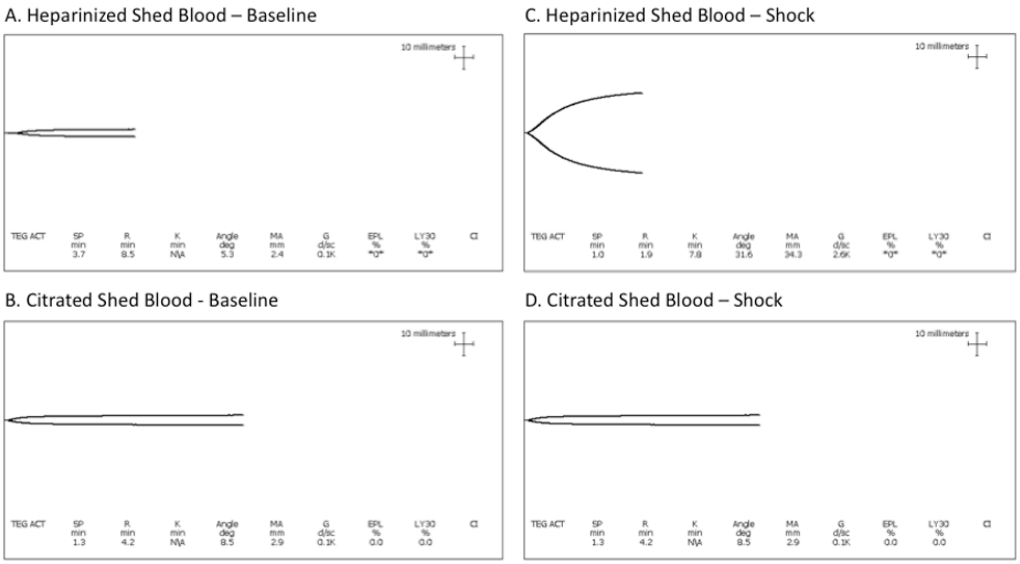

Fig. 3.

Representative tracings of the modified PlateletMapping assay. A, Baseline tracing of heparinized blood collected at the beginning of the experiment with a *G* value = 0.1 d/sc. B, Baseline tracing of citrated blood collected at the beginning of the experiment with a *G* value = 0.1 d/sc. C, Heparinized SB collected during shock had a significant increase in platelet function as evidenced by a *G* value of 2.6 d/sc, representing GPIIb/IIIa receptor activation, compared with baseline. D, Citrated SB collected during shock had no increase in platelet activation with a *G* value of 0.1 d/sc, identical to baseline.

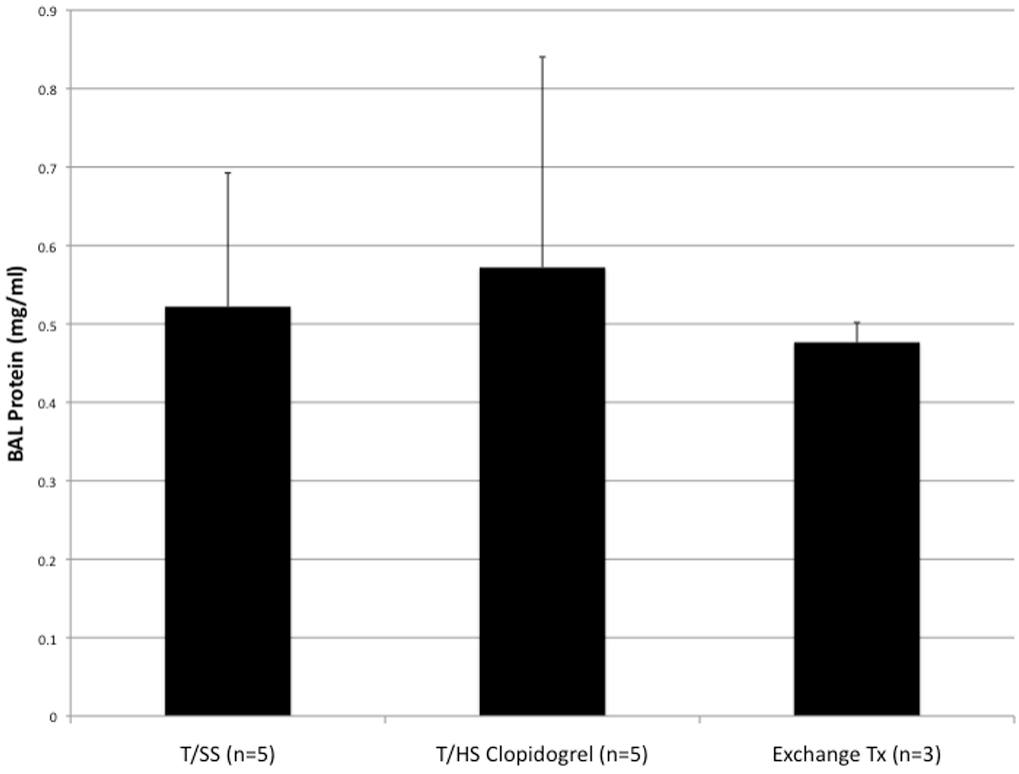

Fig. 4.

Bronchoalveolar lavage protein measured in T/SS, T/HS clopidogrel + heparin, and exchange transfusion groups. There are no significant changes seen in the BAL protein values between T/SS and the T/HS clopidogrel + heparin group or exchange transfusion group.

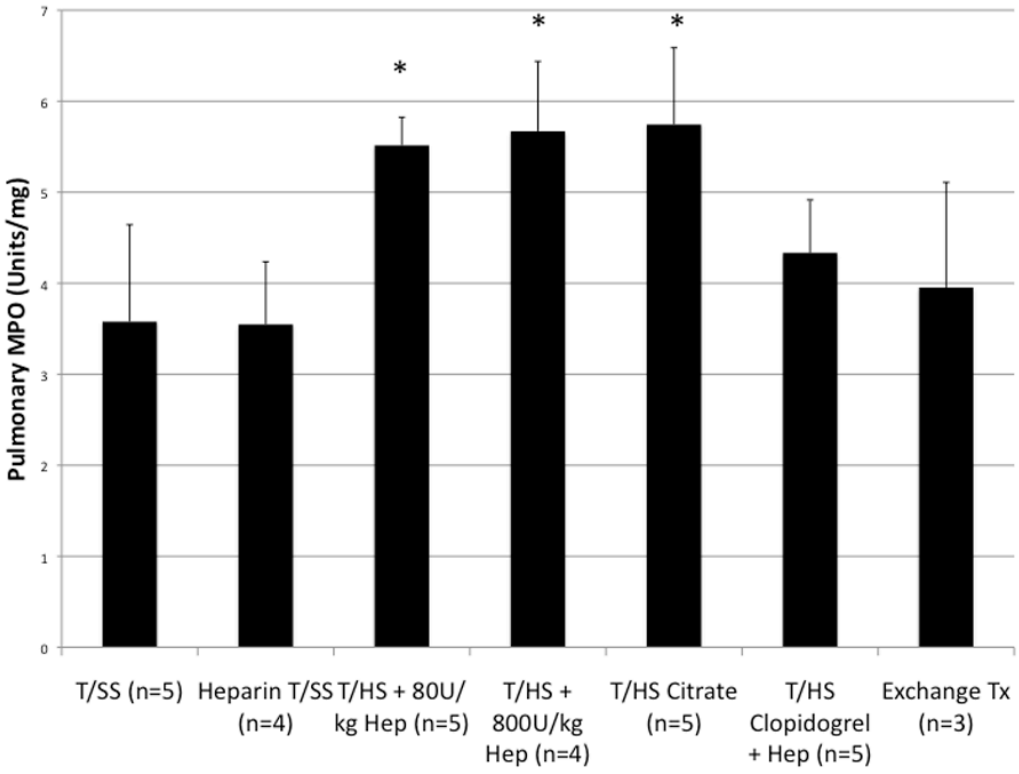

Fig. 5.

Myeloperoxidase activity from homogenized lung tissue measuring pulmonary PMN accumulation. Animals undergoing T/HS with the return of SB had increased pulmonary PMN accumulation except the T/HS clopidogrel + heparin group. **P*< 0.001 compared with T/SS group.

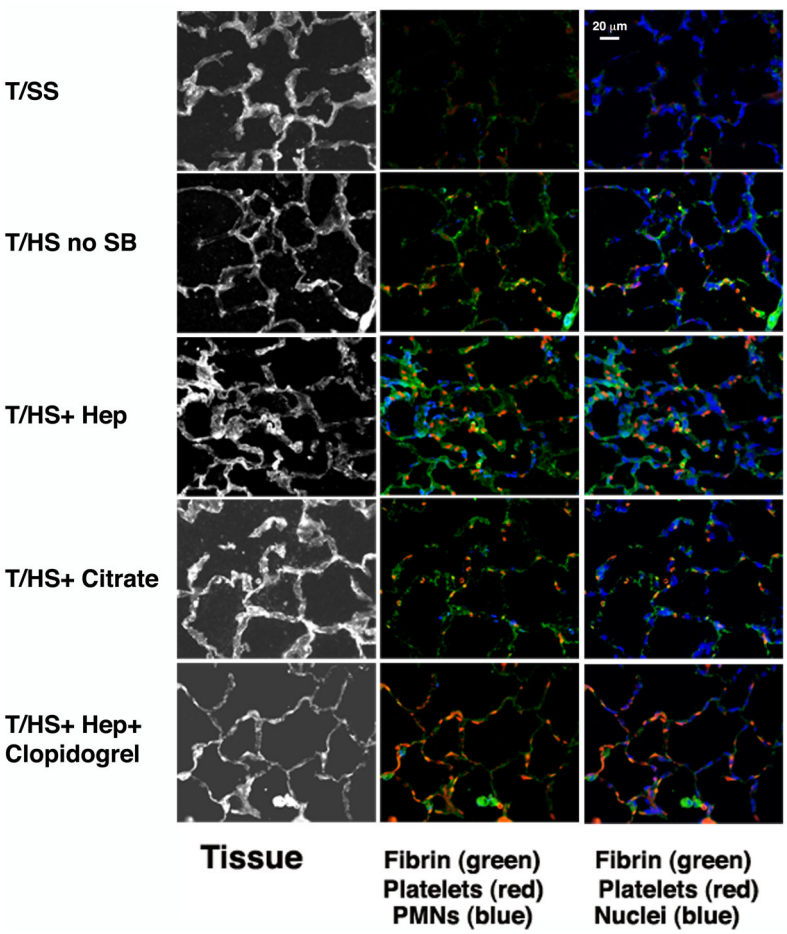

Fig. 6.

Fibrin, platelet, and neutrophil immunofluorescence in lung tissue. Mean sum intensities are displayed in Figure 7. Trauma/sham shock group has minimal fibrin, platelets, and neutrophils. Trauma/hemorrhagic shock (no SB) group has increased fibrinogen, platelets, and neutrophils, and the T/HS+SB group has the most significant increase. The T/HS citrate and T/HS clopidogrel + heparin groups have no significant change compared with T/SS. Although the T/HS clopidogrel + heparin group visually has more platelet immunofluorescence, the mean sum intensity is not significant compared with the T/SS group.

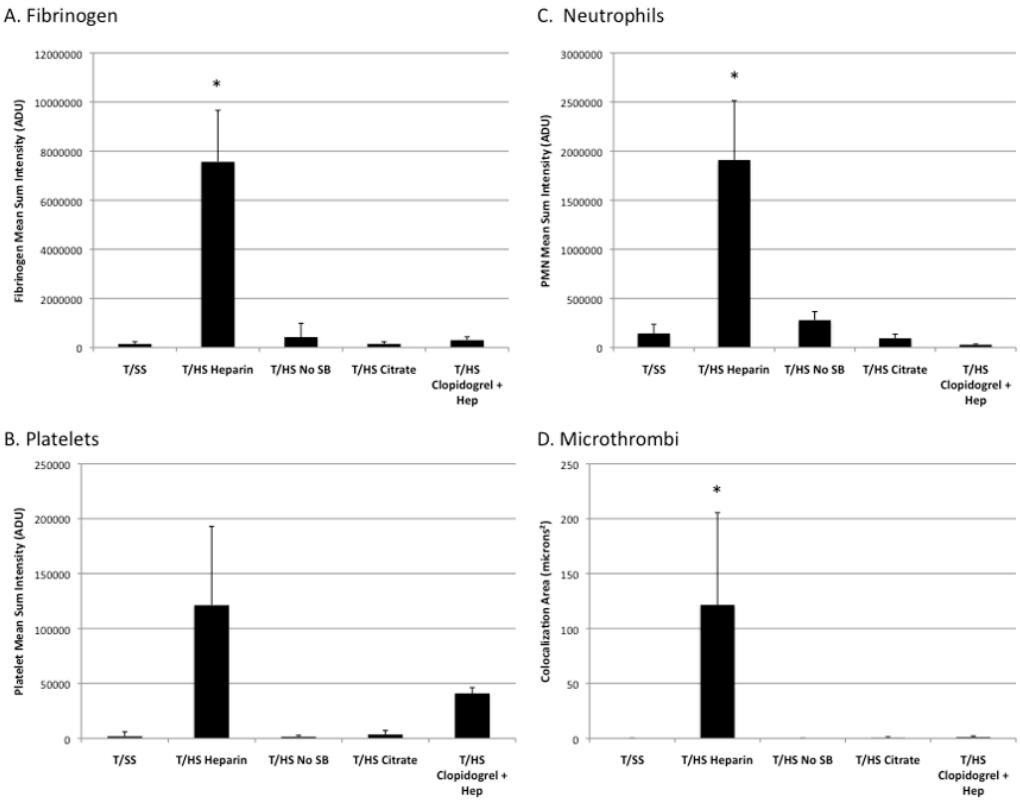

Fig. 7.

Fibrinogen, platelet, and neutrophil immunofluorescence of control, T/SS, T/HS, T/HS no SB, T/HS citrate, and T/HS clopidogrel + heparin group lungs. A, Fibrinogen immunofluorescence is significantly higher only in the T/HS heparin group. B, Platelet immunofluorescence is higher in the T/HS heparin group, but this difference is not significant. C, Neutrophil immunofluorescence is significantly higher in the T/HS group. D, Colocalization of fibrinogen, platelets, and neutrophils, representing microthrombi, was significant only in the T/HS group. *$P < 0.001$.