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Platelet releasates mitigate the endotheliopathy of trauma

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Abstract

BACKGROUND: Platelets are well known for their roles in hemostasis, but they also play a key role in thromboinflammatory pathways by regulating endothelial health, stimulating angiogenesis, and mediating host defense through both contact dependent and independent signaling. When activated, platelets degranulate releasing multiple active substances. We hypothesized that the soluble environment formed by trauma platelet releasates attenuates thromboinflammation via mitigation of trauma induced endothelial permeability and metabolomic reprogramming.

METHODS: Blood was collected from injured and healthy patients to generate platelet releasates and plasma in parallel. Permeability of endothelial cells when exposed to trauma platelet releasates (TPR) and plasma (TP) was assessed via resistance measurement by Electric Cell-substrate Impedance Sensing (ECIS). Endothelial cells treated with TPR and TP were subjected to mass spectrometry-based metabolomics.

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RESULTS: TP increased endothelial permeability, whereas TPR decreased endothelial permeability when compared to untreated cells. When TP and TPR were mixed *ex vivo*, TPR mitigated TP-induced permeability, with significant increase in AUC compared to TP alone. Metabolomics of TPR and TP demonstrated disrupted redox reactions and anti-inflammatory mechanisms.

CONCLUSION: TPRs provide endothelial barrier protection against TP-induced endothelial permeability. Our findings highlight a potential beneficial action of activated platelets on the endothelium in injured patients through disrupted redox reactions and increased antioxidants. Our findings support that soluble signaling from platelet degranulation may mitigate the endotheliopathy of trauma. The clinical implications of this are that activated platelets may prove a promising therapeutic target in the complex integration of thrombosis, endotheliopathy, and inflammation in trauma.

LEVEL OF EVIDENCE: Prognostic/Epidemiological, Level III

Keywords

Endotheliopathy; mass spectrometry; metabolomics; platelet releasates; trauma

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Much of the morbidity and mortality associated with injury results from the thromboinflammatory milieu of trauma characterized by bleeding, coagulopathies, and inflammatory complications (1). This thromboinflammatory state occurs via both cellular interactions and the release of mediators from damaged cells and tissue resulting in dysregulation in endothelial function (2-4). The endothelium is a critical interface between the vascular compartment and tissues, and functions to maintain vascular homeostasis including prevention of thrombosis and maintenance of hemostasis. In the post-trauma inflammatory milieu, there is a transition from localized injury to a systemic reaction characterized by a loss of endothelial barrier function, vascular permeability, and a pro-inflammatory state (1). It is known in many diseases that an important mediator of endothelial regulation are platelets through both cellular and noncellular signaling (5, 6).

Platelets are central contributors to vascular microenvironments and are key players in a multitude of physiological and pathological processes (7-9). They contain a variety of biomolecules, approximately 1100 different proteins, which are triggers for hemostasis and support cell survival, endothelial barrier integrity, and inhibit cell apoptotic pathways (10-14). The contents released on platelet degranulation, or platelet releasates, create microenvironments of biologically active compounds and proteins which allow for efficient delivery of growth factors and immune modulators. The isolated effect of platelet releasates on the endotheliopathy of trauma has yet to be explored.

As endotheliopathy is a well-characterized contributor to post trauma sequelae, targeted therapeutics to mitigate this effect is critical. In addition, severe metabolic disturbance is an established hallmark of major trauma, and the endothelium plays a significant role. The production of reactive nitrogen species (RNS) and reactive oxygen species (ROS) within endothelial cells is essential for numerous crucial signaling pathways related to cell survival, proliferation, activation, stress response, cell motility, vasodilation, and

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angiogenesis (15-16). To maintain redox homeostasis, endothelial cells produce antioxidant enzyme systems to eliminate intracellular oxidants.

Understanding the relationship between platelet signaling and the inflammatory trauma milieu requires a comprehensive analytical approach, which to date has not been explored. Recent advances in metabolomics technologies have enabled the quantitative detection of hundreds of metabolites in cells, tissues, and plasma. While advanced bioinformatics methods applied to omics data has elucidated molecular mechanisms that drive complications after trauma (17, 18). Through these methods, we seek to identify a comprehensive characterization of the effect of platelet releasates on the endotheliopathy of trauma. We hypothesized that the soluble environment formed by trauma platelet releasates attenuates thromboinflammation via mitigation of trauma induced endothelial permeability and metabolomic reprogramming.

MATERIALS AND METHODS

Patient Selection and Sample Collection and Processing

Samples were selected from patients who were 18 years of age or older and had been prospectively enrolled in a long-standing study of coagulation and inflammation in injury (IRB# 19-28933). The STrengthening the Reporting of OBservational Studies in Epidemiology (STROBE) checklist was used to ensure abidance to the Enhancing the QUAlity and Transparency Of Health Research (EQUATOR) guidelines for reporting observational studies (Supplemental Digital Content, STROBE Checklist, http://links.lww.com/TA/D263). These patients had met criteria for the highest level of trauma activation at an urban, level 1 trauma center and the origin blood that was collected to create platelet releasates and plasma was collected prior to any therapies including blood product transfusion. Platelet releasate was generated following established procedures (5, 19). Whole blood from trauma patients underwent centrifugation at 200xg for 10 minutes, and the resulting platelet-rich plasma was further centrifuged at 1000xg for 10 minutes in the presence of a 1:10 acid citrate dextrose solution A anticoagulant. The platelets obtained were resuspended in HEPES-buffered Tyrode's solution containing 1mM Prostaglandin-E1 and quantified. After pelleting the platelets once more, they were resuspended at a concentration of 5x10^5/mL in HEPES-buffered Tyrode's supplemented with 2mM CaCl2 to facilitate content release. The resultant platelet releasate was collected and stored at −80°C. In parallel, plasma samples were generated and stored at −80°C. In addition, whole blood from healthy donors underwent identical processing to establish control treatment groups.

Assessment of Permeability

Electric cell-substrate impedance sensing (ECIS®) measures permeability changes between cells in real time in primary human umbilical vein endothelial cells (HUVECs) by measuring resistance between cells via gold electrodes coated on the bottom of each well, and therefore permeability. Resistance to flow of current over the confluent HUVECs is an indicator of endothelial barrier integrity. HUVECs were cultured and grown to confluence prior to transfer to a standard, 96-well, 10idf plate. The gold electrodes ran an alternating current at 4,000 Hz, where the impedance of cell membranes is high, allowing most of

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the current to flow under the cells and through the tight spaces between the cells. Growth curves, based on the increased resistance in each ECIS plate well, were then obtained prior to commencement of any experiment. The normalized resistance, and thus changes to permeability, was measured in real-time on ECIS over an hour.

Plasma Permeability

20 μL samples of plasma were applied to each well, confluent with HUVECs, containing 180 μL of un-supplemented, VascuLife ® media. Resistance, and therefore permeability changes between cells, were then recorded for 30 minutes. Recombinant factor II (F2), or thrombin, was used as a positive control. A group of untreated HUVECs served as a negative control.

Platelet Releasate Permeability

Endothelial cells were cultured and grown to confluence prior to transfer to a standard, 96-well, 10idf plate. The alternating current of the gold electrodes was again set at 4,000 Hz. HUVECs in supplemented media were used as a negative control and a separate group of HUVECs were treated with thrombin to be used as our positive control. We then traced the normalized resistance, and thus permeability changes, in real time via ECIS for one-hour minutes after addition of our treatment groups, trauma platelet releasates, healthy platelet releasates, trauma platelet releasates in combination with trauma plasma, and healthy platelet releasates in combination with trauma plasma. We quantified permeability changes in the control and treatment groups by calculating the area under their curves. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a post hoc analysis using Tukey's multiple comparison's test.

Sample Preparation for Metabolomic Analysis

For downstream metabolomic testing, HUVECs were grown to confluence on 6-well plates. Trauma plasma, trauma platelet releasates, trauma platelet releasates in combination with trauma plasma, and healthy platelet releasates in combination with trauma plasma were added to the HUVECs. The supernatant and pellet were collected at 1 and 4 hours from addition of treatment groups, frozen and sent for multi-omic analysis. Metabolites were extracted from our samples for liquid chromatography-mass spectrometric analysis.

Mass Spectrometry (MS)-based Omics Analysis

For metabolomics extracts, cold 5:3:2 MeOH:ACN:H2O (v/v/v) solution is added to samples. Samples are then vortexed vigorously for 30 minutes at 4°C, then centrifuged for 10 minutes at 18,213 rcf. Samples were extracted at different ratios based on the composition. For our HUVECs, Cells were extracted $2x10^6$ cells/mL in 5:3:2 MeOH:ACN:H_2O (v/v/v). Extracts were run for metabolomics. Our plasma samples were separated into 20 μL aliquots for metabolomics. Aliquots were extracted at 1:25. Platelets were separated at 10 μL aliquots for metabolomics, and aliquots were extracted at 1:10. The supernatant from our HUVECs were separated into 20 μL aliquots for metabolomics, and aliquots were extracted at 1:25. Using 5 μL injection volumes, metabolites were resolved using a high-throughput 1-minute gradient method as previously described. (20), (21) For

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all samples, quality control was assessed as using technical replicates run at beginning, end, and middle of each sequence as previously described. (22) Files were converted to.mzXML using RawConverter. Metabolites were assigned and peaks were integrated using Maven (Princeton University) in conjunction with the KEGG database and an in-house standard library.

Statistical Analysis

Metabolite raw intensity values were auto-scale normalized, then compared using partial least squares-discriminant analysis (PLS-DA) and analysis of variance (ANOVA) methods in MetaboAnalyst software (23). PLS-DA was employed to determine whether distinct metabolomic signatures existed between conditions. Metabolites responsible for causing different signatures, in the PLS-DA model, were determined by calculating variable importance in projections (VIP) scores. 2-way ANOVA was employed to test whether metabolites showed significant differences as a function of the variables time and experimental condition. The top 50 significant metabolites, as measured by two-way ANOVA were visualized by heatmaps using MetaboAnalyst. To further compare the metabolite raw intensities in this study, statistical significance was measured by either unpaired t-test or Tukey's multiple comparison's test where appropriate, using Prism Graphpad software. The alpha value 0.05 was considered statistically significant.

RESULTS

Electric Impedance Measurements

Permeability tracings were obtained for HUVECs treated with *14 ex vivo* plasma and 14 platelet releasate samples collected from patients requiring the highest level of trauma activation at an urban Level I Trauma Center. These patients were mostly male (71%), white (36%) and middle aged (median age 52). They had a median platelet count of 288, base excess (BE) of –2.5, and 64% sustained blunt trauma. There were three deaths in this group (31%). Supplemental Digital Content 2, http://links.lww.com/TA/D789, shows demographic and clinical data for patients. Application of each treatment group on HUVECs induced changes in permeability that were statistically significant when compared to each other and the control groups (Fig. 1a). Trauma plasma from injured patients added to HUVECs demonstrates a decrease in resistance which corresponds to an increase in endothelial permeability consistent with prior work (Fig. 1a) (24). Trauma platelet releasates alone on HUVECs demonstrated a decrease in permeability from the control (Fig. 1a). When trauma platelet releasates in combination with trauma plasma were tested, there was a decrease in trauma plasma induced permeability Additionally, healthy platelet releasates in combination with trauma plasma mitigated the trauma plasma induced permeability (Fig. 1a). Overall, trauma plasma resulted in increased permeability and trauma platelet releasates in combination with trauma plasma resulted in significantly decreased permeability of normalized transendothelial electrical resistance compared to trauma plasma (Fig. 1b).

Metabolomics

HUVEC cells treated with control, trauma plasma, trauma platelet releasates, trauma platelet releasates in combination with trauma plasma, and healthy platelet releasates in combination

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with trauma plasma were collected after 1 or 4 hours of incubation and resulting cell pellets were subjected to MS-based metabolomics analysis (Fig. 2). PLS-DA demonstrated distinct metabolomic signatures in HUVEC cells treated with trauma plasma, trauma platelet releasates, trauma platelet releasates in combination with trauma plasma, and healthy platelet releasates in combination with trauma plasma (Fig. 3). Based on analytes in the top 15 VIP, relative levels of metabolites healthy platelet releasates in combination with trauma plasma were consistent with aberrant carnitine and fatty acid metabolism, arginine and proline metabolism and nucleotide synthesis at one hour (Fig. 3a). At the four-hour timepoint, relative levels of metabolites in healthy platelet releasates in combination with trauma plasma were consistent with disrupted redox reactions and altered sugar metabolism (Fig. 3b). The top 50 metabolites that were significantly different as a function of time and experimental condition, as measured by 2-way ANOVA, were visualized using a heatmap which demonstrated differences between control and treatment groups over the monitored time course (Supplemental Digital Content 3, http://links.lww.com/TA/D790). This initial analysis confirmed the existence of a distinct metabolic signature associated with trauma platelet releasates in combination with trauma plasma, and healthy platelet releasates in combination with trauma plasma.

The metabolomic changes revealed platelet releasate alterations in specific biochemical pathways and processes. Specific to trauma platelet releasates in combination with trauma plasma treatment group, there was an increase in antioxidants over time in the effluent from endothelial cells (Fig. 4a). In our healthy platelet releasates in combination with trauma plasma in the effluent from endothelial cells there was an increase in antioxidants over time as well (Fig. 4b). When we compare the raw intensity between our control and treatment groups specifically for redox reactions, we demonstrate an increased response in antioxidants in the treatment groups that contained either trauma platelet releasates or healthy platelet releasates. In our control group compared to our cells treated with trauma plasma for four hours, there was no difference in antioxidants between the two groups in the effluent from endothelial cells (Fig. 5). However, there was a difference between trauma plasma and healthy platelet releasates in combination with trauma plasma in the endothelial cell effluent with antioxidants (Fig. 5). There was no difference in healthy platelet releasates in combination with trauma plasma in levels of 5-oxoproline or glutathione disulfide. There was no difference in antioxidant levels in trauma plasma and trauma platelet releasates in combination with trauma plasma.

DISCUSSION

Here we present the first data focused on the molecular impact of platelet releasates on the circulating metabolome of trauma patients. We showed that platelet releasates can mitigate the endotheliopathy of trauma by restoring and protecting endothelial barrier function and changing the metabolomic milieu. Further, our data identifies cellular and subcellular processes associated with the cytoprotective effect of platelet releasates. Platelet releasates from both trauma and healthy patients provide endothelial barrier protection against trauma plasma induced endothelial permeability. Our findings highlight a potential beneficial action of activated platelets on the endothelium in injured patients. The clinical implications of

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this are that activated platelets may prove a promising therapeutic target in the complex integration of thrombosis, endotheliopathy, and inflammation in trauma.

The circulating environment of hemorrhagic shock and severe injury after trauma induces endothelial permeability and damage (24-28). Endothelial cell injury following trauma predisposes the systemic environment to apoptosis and inability to maintain homeostasis. Recognition that endothelial injury is a critical initial step in the post trauma inflammatory milieu, supports those therapeutics targeting the endothelial disruption is crucial. We found that platelet releasates have endothelial barrier protection in our *ex-vivo* ECIS model, and metabolomics suggest redox mechanisms play a role. The treatment of endothelial cells exposed to trauma plasma with platelet releasates demonstrates an increase in redox mechanisms suggesting that platelet releasates may contain or promote the production of antioxidants for cytoprotection.

Increased exposure to stress among trauma patients frequently leads to redox adaptations linked with elevated levels of oxidative stress. Among the metabolites detectable through metabolomics, there are specific ones that aid in comprehending the mechanisms behind reactions leading to the generation of ROS. ROS, a byproduct of aerobic metabolism, encompass the superoxide anion, hydrogen peroxide, and hydroxyl radicals, each exhibiting unique chemical reactivity with biological targets such as lipids, proteins, and DNA. Typically, ROS are associated with oxidative stress conditions, a detrimental process affecting lipids, proteins, and DNA, implying that ROS contribute to various pathologies. While not explicitly characterized after trauma, oxidative stress can be considered a state in which ROS exert influence, leading to substantial modifications in the overall cellular redox status. These alterations could result in varied and potentially impactful effects on essential metabolic processes necessary for facilitating recovery in individuals experiencing trauma.

To counterbalance the generation of reactive oxygen species (ROS), the organism employs antioxidant enzymes along with intracellular molecules like glutathione, present in both reduced (GSH) and oxidized (GSSG) states (29). Additionally, there are circulating antioxidants such as uric acid, ascorbate, α-tocopherol, thiols, and bilirubin (16, 30, 31). Our study was consistent with increased antioxidants in the endothelial cells exposed to healthy platelet releasates in combination with trauma plasma as a response to the oxidative stress from the cell's exposure to trauma plasma. Glutathione acts as a potent antioxidant, scavenging ROS and RNS and preventing their harmful effects on endothelial cells (29). By neutralizing these reactive species, glutathione helps reduce oxidative stress and minimize damage to the endothelial barrier. Additionally, glutathione helps regulate endothelial cell functions such as apoptosis, angiogenesis and cellular growth thus impacting endothelial cell fate (30, 31). Ascorbate acts in a similar mechanism as a potent antioxidant but also contains anti-inflammatory function as well (32). Because reduced levels of antioxidants are associated with severe disease and damage, introducing platelet releasates from healthy patients could be critical in protecting the vulnerable endothelial barrier.

Amino acids are crucial regulators in maintaining homeostasis by modulating endothelial cell function and play important roles in response to stress, anti-inflammatory mechanisms, redox reactions, and regulation of apoptosis. Taurine, a non-essential amino acid,

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additionally plays a role in cytoprotection through antioxidant and anti-inflammatory mechanisms. Initially, taurine serves as a validated anti-inflammatory agent, counteracting the oxidant hypochlorous acid produced by neutrophils (33). Glutamine is crucial in high stress situations such as trauma due to its cytoprotective effects and its role as a precursor for various metabolites, components of the Krebs cycle and nucleotides (AMP, purines, and pyrimidines) (34). Additionally, it contributes to the activation of the chaperone function through the heat shock protein response and supports antioxidant defense through glutathione mediation (35). Glutamine is often decreased during critical disease conditions such as in trauma and its decrease in availability is linked to impaired immune function. In these conditions, the availability of glutamine is critical in promoting recovery of endothelial cells to offer protection via glutamine-glutathione axis. Thus, the presence of increased glutamine in our trauma plasma with releasate treatment groups in comparison to our trauma groups suggests that releasates may be contributing to this exaggerated alteration in the redox balance. Like glutamine, methionine performs an essential role in stimulating glutathione synthesis by decreasing ROS accumulation (36).

Previous studies have demonstrated that treatment of endothelial cells with serine following exposure to oxidative stress results in heightened expression of antioxidant factors like nuclear factor-erythroid-related 2 factor (NRF2), heme oxygenase-1 (HO-1), and nitric oxide (37). This, in turn, enhances endothelial cell viability and survival, indicating the potential role of serine in modulating the response to oxidative stress. Nrf2 functions as a redox-sensitive transcription factor that stimulates the synthesis of antioxidant enzymes like HO-1 which through the metabolism of the oxidant heme, produces bilirubin and carbon monoxide that are important antioxidants (37). Additionally, studies have shown that Nrf2 plays a crucial role in safeguarding cells and tissues from inflammatory injuries (38). Additionally, serine can regulate endoplasmic reticulum activity, suppress inflammatory responses and oxidative stress, and enhance endothelial function by inhibiting homocysteine uptake by endothelial cells. In a similar mechanism to serine, alanine promotes expression of the antioxidant defense proteins HO-1 and ferritin in endothelial cells which improve endothelial dysfunction (39). Addition of platelet releasates to trauma plasma appears to mitigate the state of oxidative stress and pro-inflammatory mechanisms and suggests that platelet releasates may serve as a promising therapeutic target in the endotheliopathy of trauma.

Limitations

Our study is limited due to understanding that plasma metabolites are rather dynamic and can fluctuate due to multiple factors. Additionally, we used an *in vitro* model and while this environment is a critical step in exploring the effect on endothelial cells, it is limited in its application to a systemic representation. Further, we explored the non-cellular signaling of platelets, but the cell-cell interactions of platelets in combination with their releasates may alter these relationships. Despite these limitations, the study has highlighted several intriguing avenues for future exploration including the elucidating of dosing and dynamics of the non-cellular platelet signaling effects on thromboinflammation. Utilizing metabolomics allows further exploration of pathways, at a molecular level, under controlled and reproducible conditions using animal models. The precise roles of redox metabolism

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during the endotheliopathy of trauma can be further interrogated using pharmacological inhibition in animal models in combination with omics analyses. This study combines our endothelial permeability experiments with metabolomics and establishes the foundation for identifying and developing potential mediators in the endotheliopathy of trauma.

CONCLUSION

In summary, we detected biochemical patterns suggesting platelet releasate induced endothelial cell cytoprotection is associated with metabolic aberrancies, all in the context of redox metabolism imbalance and decreased cellular/tissue destruction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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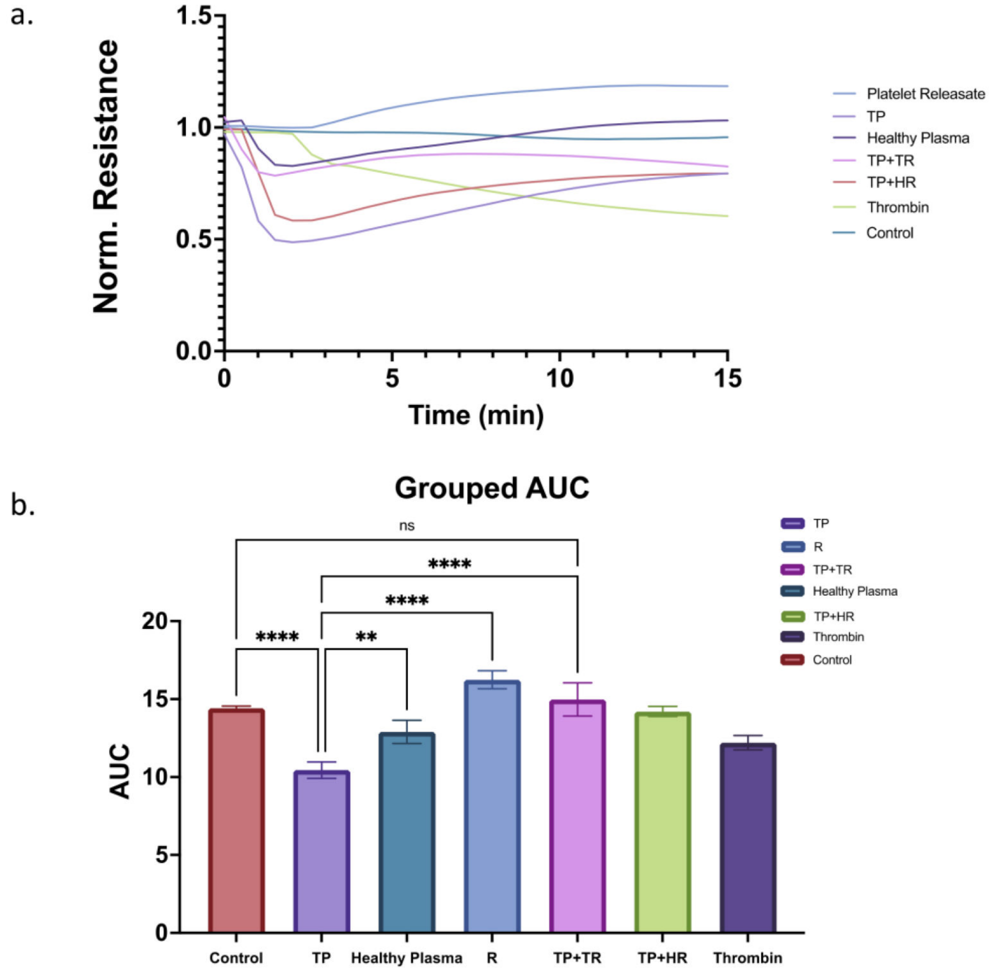

Figure 1. Endothelial Permeability Results.

Human umbilical vein endothelial cells were grown to confluence, and ECIS was used to measure endothelial permeability in response to treatment with trauma plasma (TP), Healthy Plasma, trauma platelet releasates (R), trauma platelet releasates in combination with trauma plasma (TP+TR), and healthy platelet releasates in combination with trauma plasma (TP+HR), and thrombin. Tracings are averaged normalized resistance in each well **(a).** Corresponding area under the curve (AUC) analysis for the permeability curves under the designated conditions using Kruskal-Wallis with Dunn's multiple comparisons test. Area under the curve boxplots represent AUC quantitation of changes in barrier resistance. ** indicates p-value <0.01, *** indicates p-value <0.001, **** indicates p-value <0.0001 **(b).**

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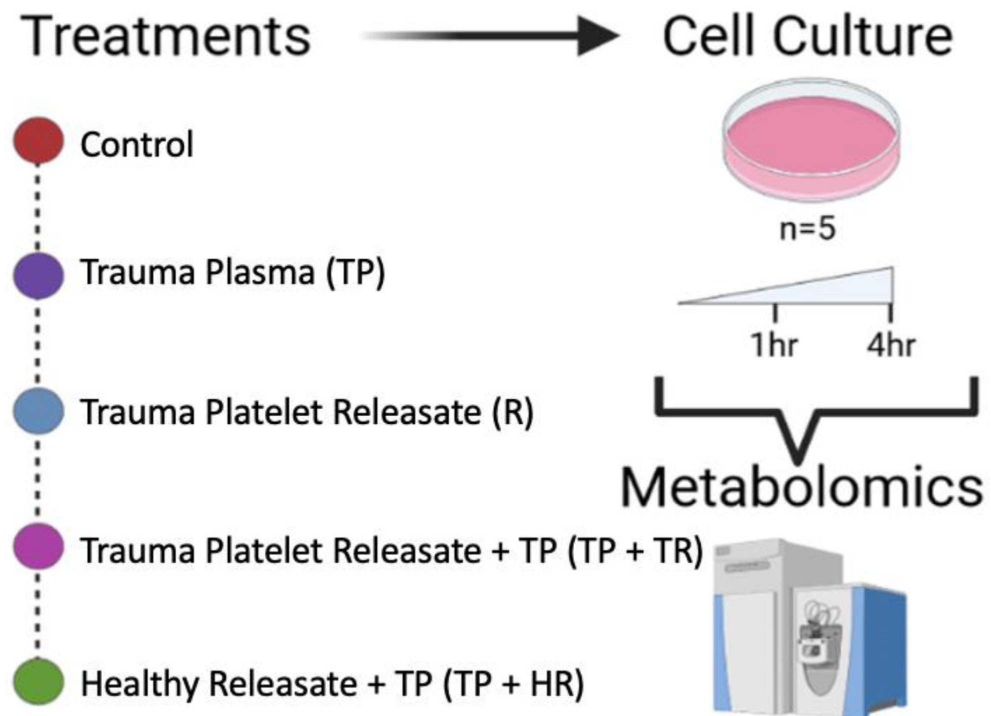

Figure 2. Overview of MS-based Metabolomics Workflow.

HUVEC cells were treated with control, trauma plasma (TP), trauma platelet releasates (R), trauma platelet releasates in combination with trauma plasma (TP+TR), and healthy platelet releasates in combination with trauma plasma (TP+HR). Cells were collected after 1hr or 4hr treatments. Metabolites were extracted from cell pellets and subjected to mass spectrometric analysis.

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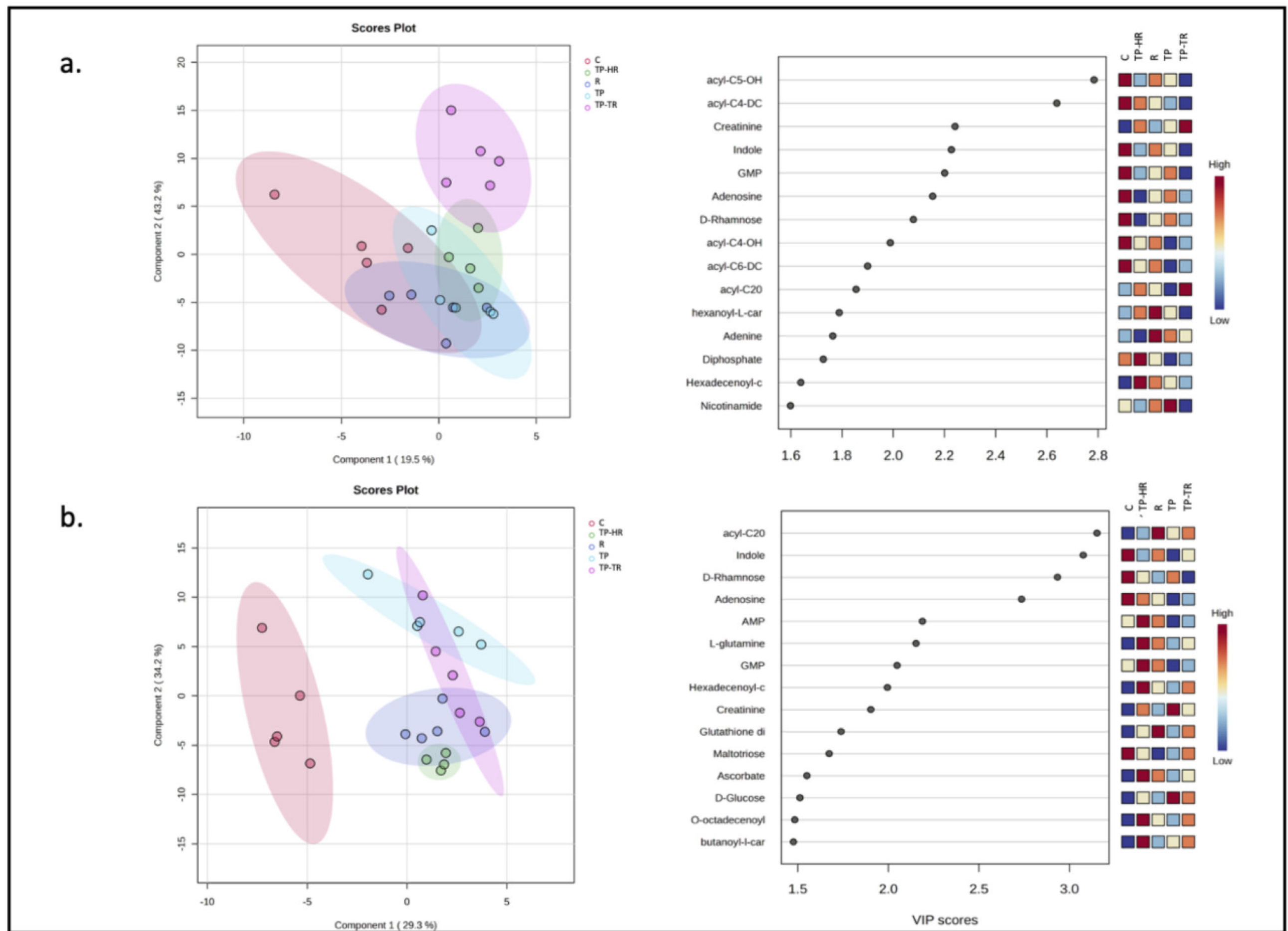

Figure 3. Diagram of Metabolomics Throughput.

PLS-DA demonstrating distinct metabolomic signatures for control (C), healthy platelet releasates in combination with trauma plasma (TP-HR), trauma platelet releasates (R), trauma plasma (TP) and trauma platelet releasates in combination with trauma plasma (TP-TR). PLS-DA and corresponding VIP plots for metabolomics at 1 hour **(a)** and 4 hour **(b)** are displayed. VIP plots display the top metabolite differentiators (important features), as indicated by VIP scores, with relative abundances for samples indicated by the colored boxes on the right of the plots. PLS-DA, partial least squares-discriminant analysis; VIP, variable importance in projections.

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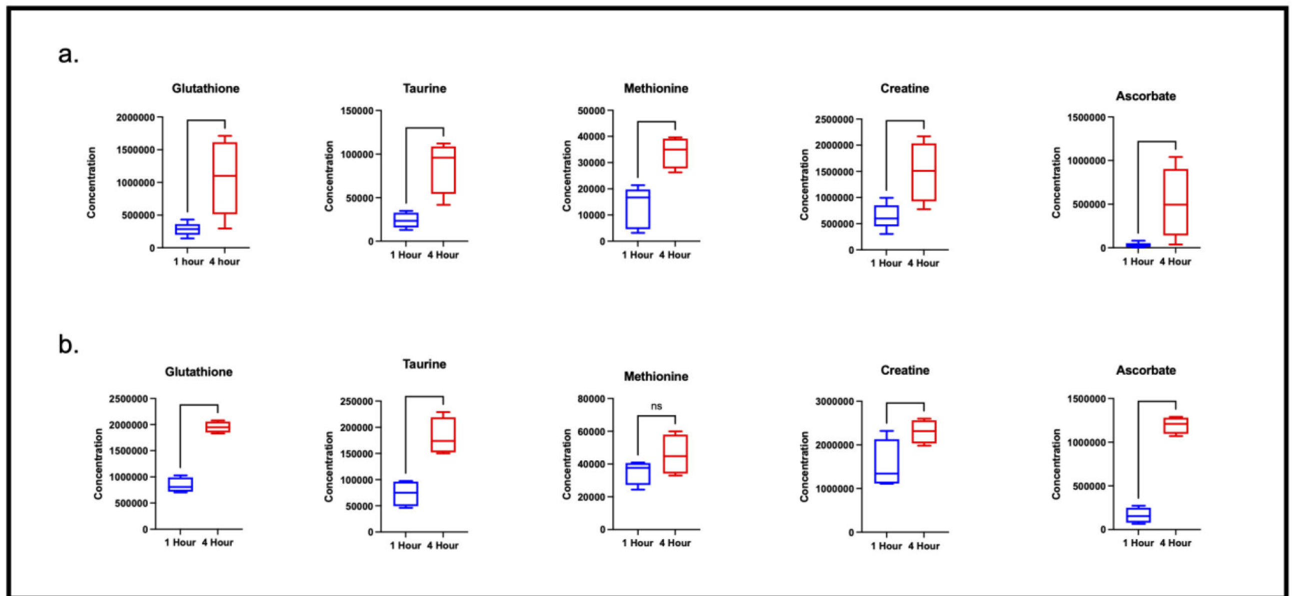

Figure 4. Redox Metabolites over Time.

Redox metabolisms significantly modulated in TP+TR over time **(a)** TP+HR over time **(b).**

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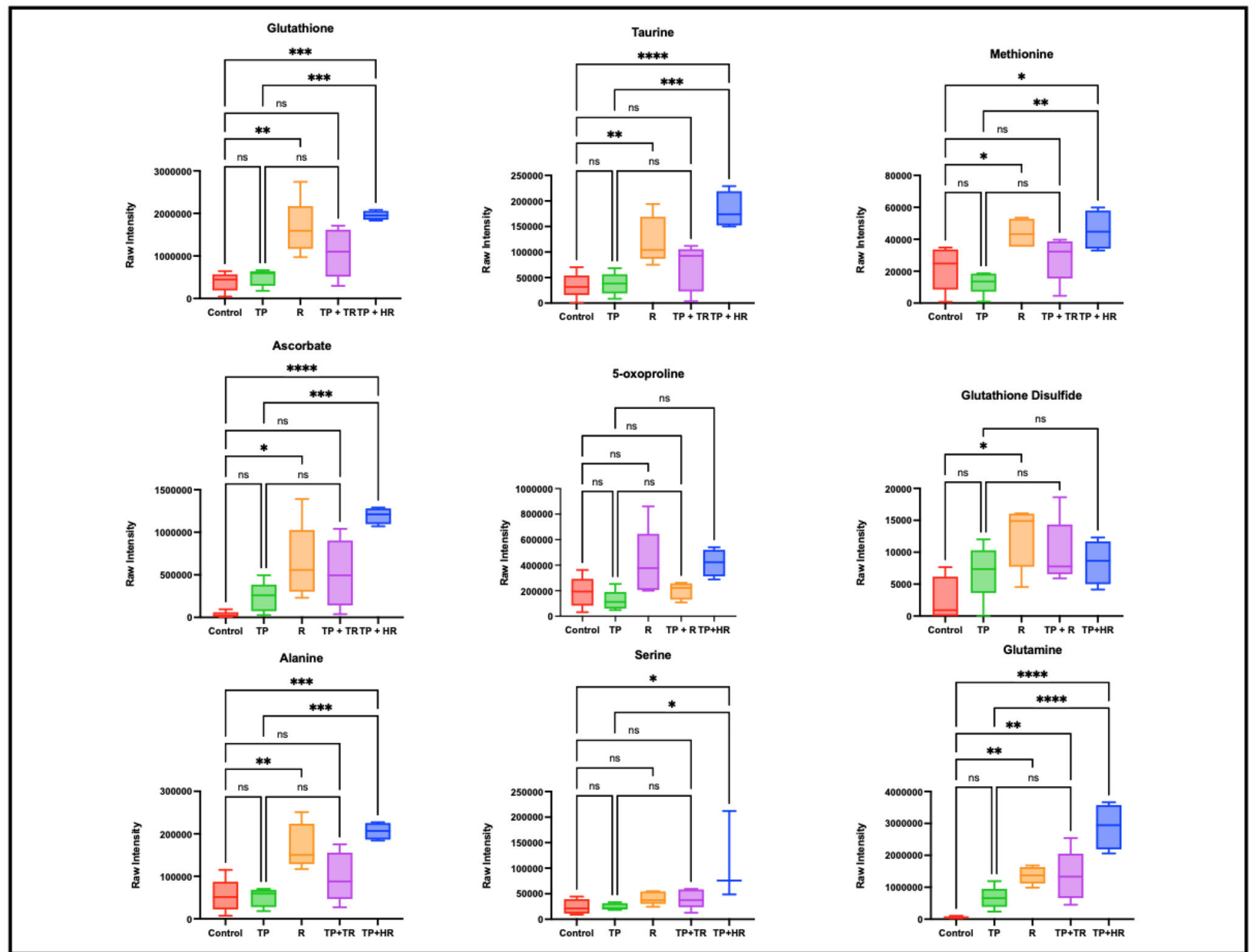

Figure 5. Redox Metabolites in Treatment and Control Groups.

Redox metabolisms significantly modulated in control and treatment groups, healthy platelet releasates in combination with trauma plasma (TP-HR), trauma platelet releasates (R), trauma plasma (TP) and trauma platelet releasates in combination with trauma plasma (TP-TR).