BRIEF REPORT

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Tissue factor release following traumatic brain injury drives thrombin generation

W. Brad Hubbard PhD[1,2,3] 🆔 🐦 | Martha M. S. Sim MS[4] 🐦 | Kathryn E. Saatman PhD[2,3] |
Patrick G. Sullivan PhD[1,3,5] | Jeremy P. Wood PhD[4,6,7] 🆔

[1]Lexington Veterans' Affairs Healthcare System, Lexington, Kentucky, USA

[2]Department of Physiology, University of Kentucky, Lexington, Kentucky, USA

[3]Spinal Cord and Brain Injury Research Center, University of Kentucky, Lexington, Kentucky, USA

[4]Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky, USA

[5]Department of Neuroscience, University of Kentucky, Lexington, Kentucky, USA

[6]Division of Cardiovascular Medicine, The Gill Heart and Vascular Institute, University of Kentucky, Lexington, Kentucky, USA

[7]Saha Cardiovascular Research Center, University of Kentucky, Lexington, Kentucky, USA

Correspondence
W. Brad Hubbard, Department of
Physiology, University of Kentucky, 445
Biomedical & Biological Sciences Research
Bldg., 741 South Limestone St, Lexington,
KY 40536, USA.
Email: bradhubbard@uky.edu

Jeremy P. Wood, Department of Molecular
and Cellular Biochemistry, University of
Kentucky, 741 S Limestone, BBSRB B359,
Lexington, KY 40536, USA.
Email: Jeremy.Wood@uky.edu

Abstract

Background: Traumatic brain injury (TBI) results in neurovascular damage that initiates intrinsic mechanisms of hypercoagulation, which can contribute to the development of life-threatening complications, such as coagulopathy and delayed thrombosis. Clinical studies have hypothesized that tissue factor (TF) induces hypercoagulability after TBI; however, none have directly shown this relationship.

Objectives: In the current study, we took a stepwise approach to understand what factors are driving thrombin generation following experimental TBI.

Methods: We employed the contusion-producing controlled cortical impact (CCI) model and the diffuse closed head injury (CHI) model to investigate these mechanisms as a function of injury severity and modality. Whole blood was collected at 6 hours and 24 hours after injury, and platelet-poor plasma was used to measure thrombin generation and extracellular vesicle (EV) TF.

Results: We found that plasma thrombin generation, dependent on TF present in the plasma, was greater in CCI-injured animals compared to sham at both 6 hours (120.4 ± 36.9 vs 0.0 ± 0.0 nM*min endogenous thrombin potential) and 24 hours (131.0 ± 34.0 vs 32.1 ± 20.6 nM*min) after injury. This was accompanied by a significant increase in EV TF at 24 hours (328.6 ± 62.1 vs 167.7 ± 20.8 fM) after CCI. Further, EV TF is also increased at 6 hours (126.6 ± 17.1 vs 63.3 ± 14.4 fM) but not 24 hours following CHI.

Conclusion: TF-mediated thrombin generation is time-dependent after injury and TF increases resolve earlier following CHI as compared to CCI. Taken together, these data

support a TF-mediated pathway of thrombin generation after TBI and pinpoint TF as a major player in TBI-induced coagulopathy.

KEYWORDS

controlled cortical impact, extracellular vesicle, phospholipid, thrombosis, tissue factor, traumatic brain injury

Essential

- Traumatic brain injury (TBI) increases the risk for blood clotting.
- Underlying factors can be identified using animal models of TBI.
- TBI results in the release of small vesicles containing the coagulation initiator tissue factor.
- The amount and duration of tissue factor release depends on the type and severity of injury.

1 | INTRODUCTION

Traumatic brain injury (TBI) leads to a myriad of secondary pathology propagated by the initial head insult. Early blood-brain barrier (BBB) disruption initiates mechanisms of thromboinflammation[1] and TBI-induced coagulopathy.[2] Coagulopathy manifests as disseminated intravascular coagulation, producing systemic blood clots that can cause secondary cerebral injury and ischemia after TBI.[3] Patients with TBI are at higher risk of blood clot formation.[4] TBI-induced coagulopathy is common in penetrating head injuries[5] but is also observed following blunt head trauma.[6] The presence of coagulopathy following isolated TBI is strongly associated with poor patient prognosis.[7]

Limitations of clinical practice in treating TBI-induced coagulopathy highlight the urgent need to identify new therapies.[8] The creation of experimental models that recapitulate clinical findings will help identify the specific etiology related to delayed thrombosis. Further, understanding of the pathobiology of coagulopathy and hypercoagulability in these models will inform researchers of potential therapeutic targets to reduce secondary brain injury and improve outcomes following TBI and will aid in identifying biomarkers for predicting which patients are most at risk for thrombosis.

Following clinical TBI, procoagulant microparticles containing phosphatidylserine (PSer) and tissue factor (TF) are released into the blood early after injury.[9] In clinical TBI plasma samples, microparticles derived from endothelial cells are more highly elevated than those from platelets or leukocytes.[10] It has been proposed that TF drives hypercoagulability following clinical TBI[5,11-13]; however, direct evidence to support this claim is lacking. Tian et al[14] showed that brain-derived extracellular vesicles (BDEVs) are released following fluid percussion injury and that BDEVs have both TF and PSer that result in thrombosis.[2] However, there is no clear evidence whether TF or PSer is the driving factor. We hypothesize that experimental TBI produces elevated levels of plasma TF, which increases the potential for thrombin generation.

For this investigation, we used two distinct preclinical TBI models to recapitulate coagulopathy of clinical TBI. Neurovascular damage is a major endophenotype of TBI and propagates pathways of

coagulopathy. The controlled cortical impact (CCI) model involves impact directly to the dura of the brain producing a relatively defined, focal cortical contusion that results in vascular injury, BBB breakdown, and acute hemorrhage at the site of impact. Intracerebral hemorrhage and vascular damage lead to local immune and inflammatory responses. The closed head injury (CHI) model involves a midline impact to the skull, which generates a relatively diffuse, mild brain injury that produces neuroinflammation and deficits in brain metabolism. CHI is nonhemorrhagic and does not produce overt cell death but can lead to diffuse vascular damage. Both models exhibit motor and cognitive deficits.[15-19] These models were used to examine thrombin production and TF activity following injury.

2 | METHODS

2.1 | Materials

Human TF (hTF) (Dade Innovin) was from Siemens Healthineers (Erlangen, Germany). Recombinant mouse factor VII was from R&D Systems (Minneapolis, MN, USA) and activated to factor VIIa (mFVIIa), following the manufacturer's protocol. Mouse TF (mTF) was from R&D Systems. Human activated factor VII (hFVIIa) and factor X (hFX) were from Enzyme Research Laboratories (South Bend, IN, USA). Phosphatidylcholine (PCho), phosphatidylethanolamine (PEth), and PSer were from Avanti Polar Lipids (Alabaster, AL, USA) and were used to prepare PCho:PEth:PSer (40:40:20) vesicles according to the protocol of Morrissey.[20] Thrombin calibrator and FluCa substrate were from Diagnostica Stago (Asnières-sur-Seine, France). Spectrozyme FXa was from BioMedica Diagnostics (Stamford, CT, USA).

2.2 | Experimental design

All of the studies performed were approved by the University of Kentucky Institutional Animal Care and Use Committee, in

compliance with the guidelines of the Association for the Assessment and Accreditation for Laboratory Animal Care, International and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.[21] All experiments were conducted using male C57BL/6J mice (2-3 months old; Jackson Laboratories, Bar Harbor, ME, USA).

The animals were housed five per cage, maintained in a 14-hour light/10-hour dark cycle, fed a balanced diet ad libitum. Animals were randomly assigned to groups, and all data analyses were performed blinded to treatment groups. Experimental groups were euthanized at either 6 or 24 hours after TBI or sham injury. For all outcomes, experiments were conducted with biological replicates of $n = 5$ to 8/group.

2.3 | Controlled Cortical Impact

The CCI procedure was performed according to past studies.[17,22] Briefly, anesthetized (2.5% isoflurane) mice were fixed with ear bars in a stereotaxic frame. After scalp incision, a 3-mm craniotomy was performed lateral to midline, and mice received a pneumatic impact (TBI-0310 Impactor, Precision Systems and Instrumentation, Fairfax, VA, USA: 1.0 mm depth; 3.5 m/s velocity; 500-millisecond dwell) directly to the left hemisphere using a 2-mm impactor tip. Sham animals received a craniotomy but no impact. Following impact, the

craniotomy was covered with absorbable hemostat (Surgicel) and a plastic cap. Mice recovered on a heating pad until the animals were fully responsive.

2.4 | Closed Head Injury

Mice were subjected to CHI based on previous studies.[23,24] Briefly, anesthetized mice (2.5% isoflurane) were fixed in a stereotaxic frame with nonrupture Zygomar ear cups (Kopf Instruments, Tujunga, CA, USA). A scalp incision was made, and mice received an impact at midline between bregma and lambda sutures using a pneumatic impactor (TBI-0310 Impactor: 2.0 mm depth; 3.5 m/s velocity; 500-millisecond dwell) fitted with a silicone tip. Sham-injured received all procedures except impact.

2.5 | Isolation of Platelet Poor Plasma

At either 6 or 24 hours after injury, animals were asphyxiated with CO_2 and up to 1 mL of blood was collected via cardiac puncture. Blood was harvested using 0.38% sodium citrate solution (supplemented with 0.2 U/mL of apyrase and 10 ng/mL of prostacyclin [Cayman Chemical, Cat. No. 18220]) in a 26G 3/8 syringe. Whole blood was centrifuged according to Hubbard et al.[25] Isolated
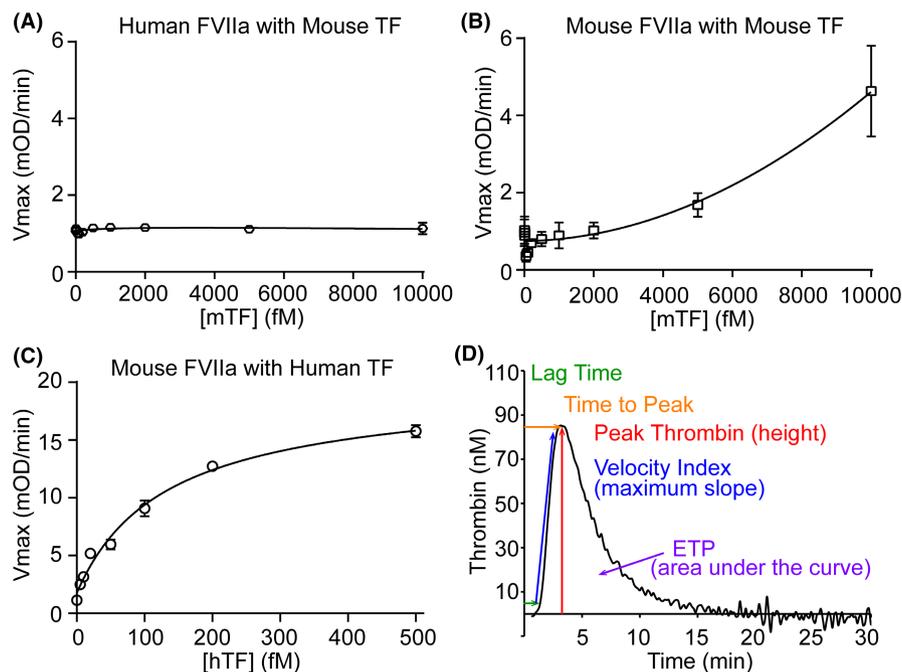

FIGURE 1 Validation of mouse TF activity and thrombin generation assays. (A, B) mTF was incubated with (A) hFVIIa (12.5 nM) or (B) mFVIIa (2.5 nM). Reactions were initiated by addition of hFX (375 nM), and cleavage of a FXa substrate (500 μM) was monitored by measuring absorbance at 405 nm for 2 h at 37°C. The maximum slope of substrate cleavage is presented (mean ± standard deviation; $n \geq 3$). (C) hTF was incubated with mFVIIa (2.5 nM), and FXa activation was measured as in (A). (D) Thrombin generation in mouse platelet-poor plasma, initiated with hTF (1 pM). Indicated are the parameters of thrombin generation determined using calibrated automated thrombography. Abbreviations: ETP, endogenous thrombin potential; FVIIa, activated factor VII; FXa, activated factor X; hFVIIa, human activated factor VII; hFX, human factor X; hTF, human tissue factor; mFVIIa, mouse activated factor VII; mTF, mouse tissue factor; TF, tissue factor; Vmax, maximum velocity of substrate cleavage
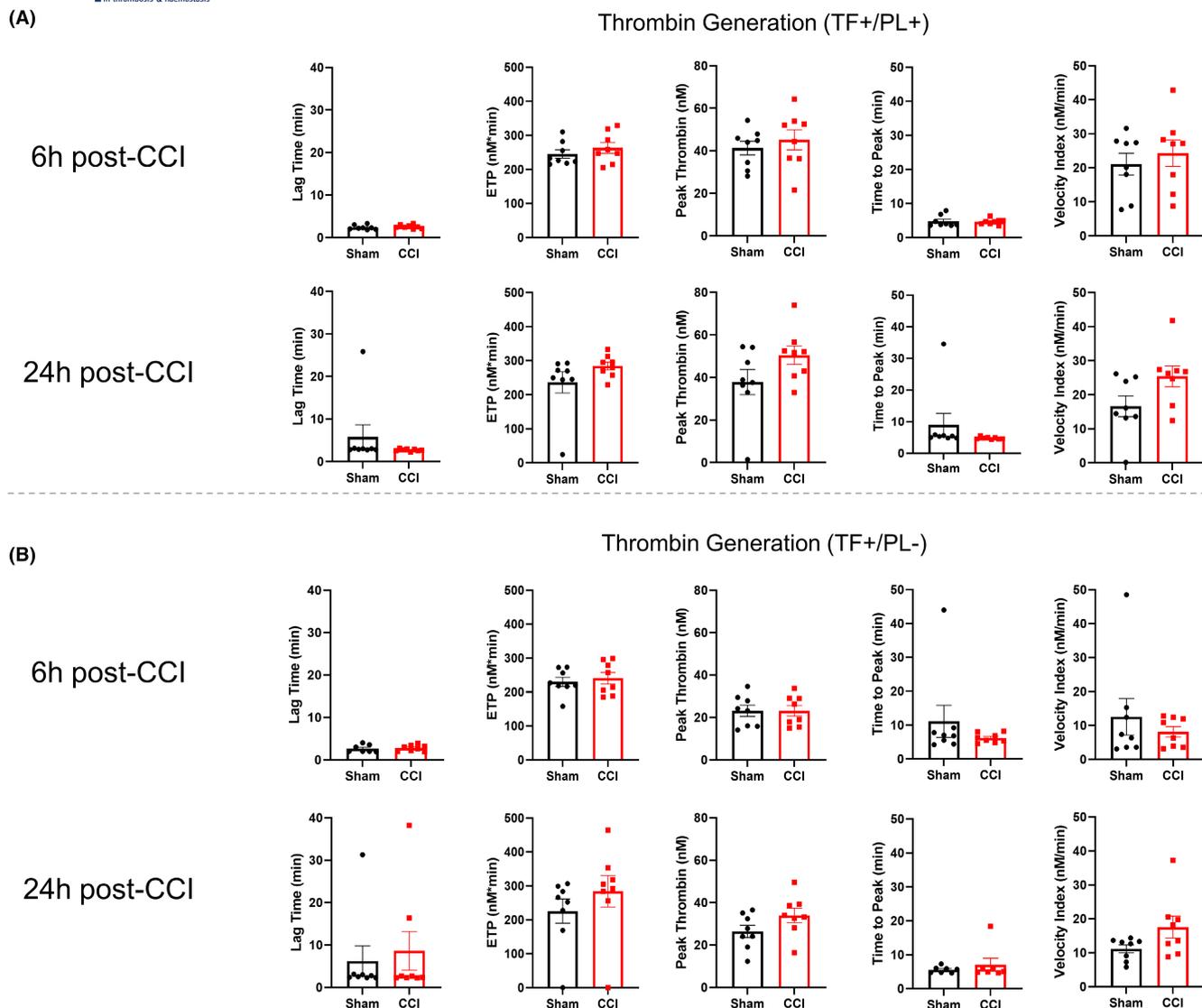
(A)

(B)

FIGURE 2 Thrombin generation is not altered after TBI when in the presence of excess TF. Mice received either sham injury or severe CCI followed by euthanasia at either 6 or 24 h after injury. PPP was assayed for thrombin generation. Lag time, ETP, peak thrombin, time to peak, and velocity index were calculated under addition of TF$^+$/PL$^+$ and TF$^+$/PL$^-$. CCI does not result in a change in any parameters in the presence of TF$^+$/PL$^+$ or TF$^+$/PL$^-$. $n = 8$/group. Mean ± standard error of the mean and data points. Abbreviations: CCI, controlled cortical impact; ETP, endogenous thrombin potential; PL, phospholipid; PPP, platelet-poor plasma; TBI, traumatic brain injury; TF, tissue factor

platelet-rich plasma was diluted 1:10 with prostacyclin (prepared at 1 mg/mL in 50 mM Tris, pH 9.5) in phosphate/glucose buffer (50 mL 1× phosphate buffered saline, 45 mg glucose [5 mM]). The samples were centrifuged at 2000 g for 6 minutes at room temperature. The supernatant (PPP) was used for subsequent analysis.

of 10 μL of calcium and fluorogenic thrombin substrate and thrombin activity was measured for up to 60 minutes, using a Fluoroskan Ascent Microplate Reader (Thermo Scientific, Waltham, MA, USA) with Thrombinoscope software (Diagnostica Stago, Parsippany, NJ, USA).

2.6 | Plasma thrombin generation

Thrombin generation was measured in PPP using calibrated automated thrombography, as described,[26] with modifications. Briefly, 40 μL of PPP was incubated with 10 μL of (i) thrombin calibrator; (ii) phospholipid vesicles (4 μM final concentration), (iii) TF (1 pM), or (iv) phospholipid vesicles and TF. Samples were incubated for 10 minutes at 37°C, thrombin generation was initiated by addition

2.7 | Plasma TF activity

TF activity was measured using an activated factor Xa (FXa) generation assay, adapted from the protocol of Hisada and Mackman.[27,28] Briefly, PPP was diluted with 1 mL HBSA (HEPES-buffered saline supplemented with 0.1% bovine serum albumin) and centrifuged (20 000 g, 45 minutes, 4°C) to isolate plasma EV-associated TF. The pellet was washed by recentrifugation with HBSA and resuspended
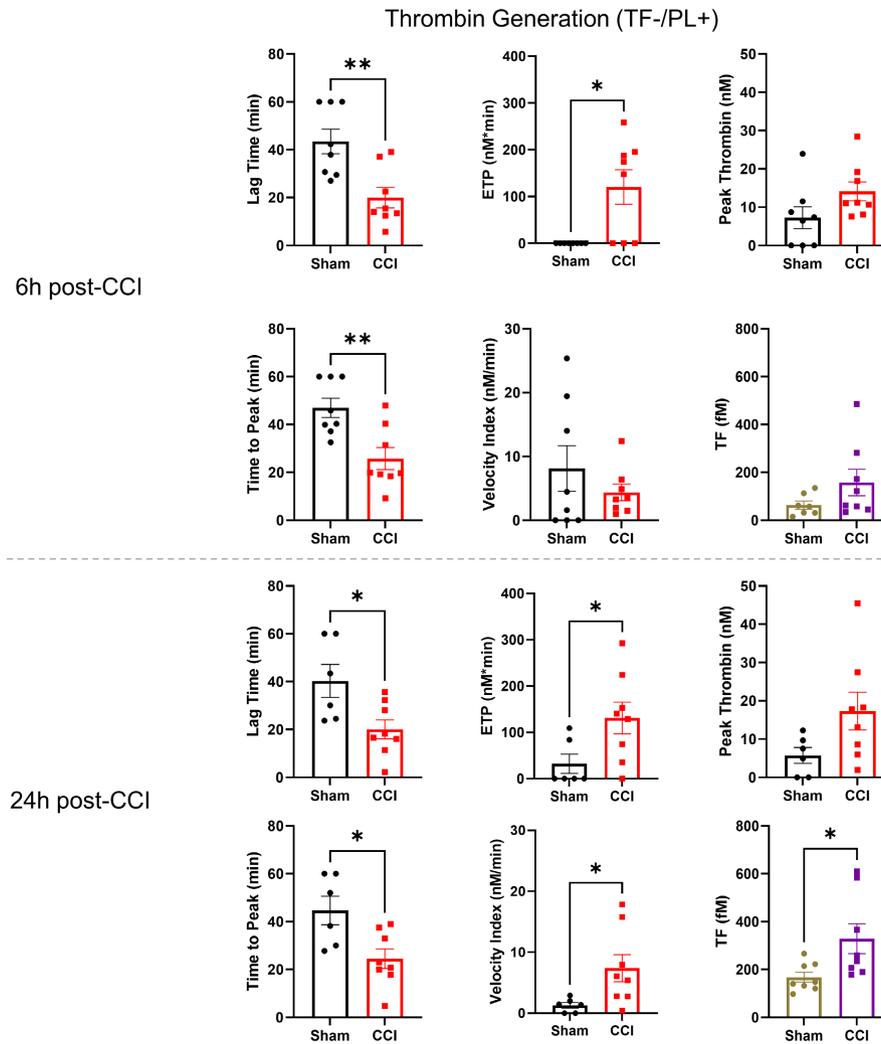
FIGURE 3 TF release following TBI drives thrombin generation. Mice received either sham injury or severe CCI followed by euthanasia at either 6 or 24 h after injury. PPP was assayed for thrombin generation. Lag time, ETP, peak thrombin, time to peak, and velocity index were calculated under addition of TF–/PL+. TF activity was also measured in the same samples. There was significantly decreased lag time and time to peak in PPP from CCI mice as compared to sham mice at both 6 h and 24 h after injury. ETP was significantly elevated in PPP from CCI mice as compared to sham mice at both 6 h and 24 h after injury. There was a significant increase in the velocity index in PPP from CCI mice as compared to that from sham mice at 24 h after injury. There was a nonsignificant increase in TF at 6 h after CCI and significant increase in TF at 24 h after CCI. 6 h: lag time **$p = 0.007$; ETP *$p = 0.03$; time to peak *$p = 0.02$. 24 h: lag time *$p = 0.04$; ETP *$p = 0.04$; time to peak *$p = 0.03$; velocity index *$p = 0.04$; TF *$p = 0.03$. $n = 6$-8/group. Mean ± standard error of the mean and data points. Abbreviations: CCI, controlled cortical impact; ETP, endogenous thrombin potential; PL, phospholipid; PPP, platelet-poor plasma; TBI, traumatic brain injury; TF, tissue factor

in 100 μL HBSA. Immediately following isolation, samples were incubated with mFVIIa (12.5 nM) and FXa chromogenic substrate (Spectrozyme FXa; 0.5 mM), reactions initiated by addition of hFX (375 nM in HBSA with 10 mM CaCl$_2$), and FXa activity monitored at 405 nm for 2 hours at 37°C. Results were compared to standards of hTF (0-500 fM) to calculate TF functional concentration. Initial experiments determined that hFVIIa (12.5 nM) does not function with mouse TF (Figure 1A), necessitating the need to use mFVIIa for subsequent experiments. Standard curves generated using mFVIIa (2.5 nM; Figure 1B,C) with mouse or human TF revealed that the standards were more sensitive and reproducible using hTF. Thus, experimental data using mouse samples are presented as the equivalent hTF activity.

2.8 | Statistical analysis

Power analysis was conducted for experimental data a priori based on effect size and expected data variance. Higher data variability was anticipated for animals undergoing craniotomy (CCI) compared to animals without craniotomy (CHI), hence expected standard deviation of 20% in CCI studies compared to 15% in CHI studies. Based on power of 0.8, 5% type I error rate, and expected mean difference of 30%, minimum group size was $n = 7$ for CCI studies and $n = 4$ for CHI studies. Statistical analysis was performed using Prism 8 (GraphPad Software, San Diego, CA, USA). For all analyses, the significance of differences was set at $p < 0.05$. Nonparametric statistics were used for thrombin generation
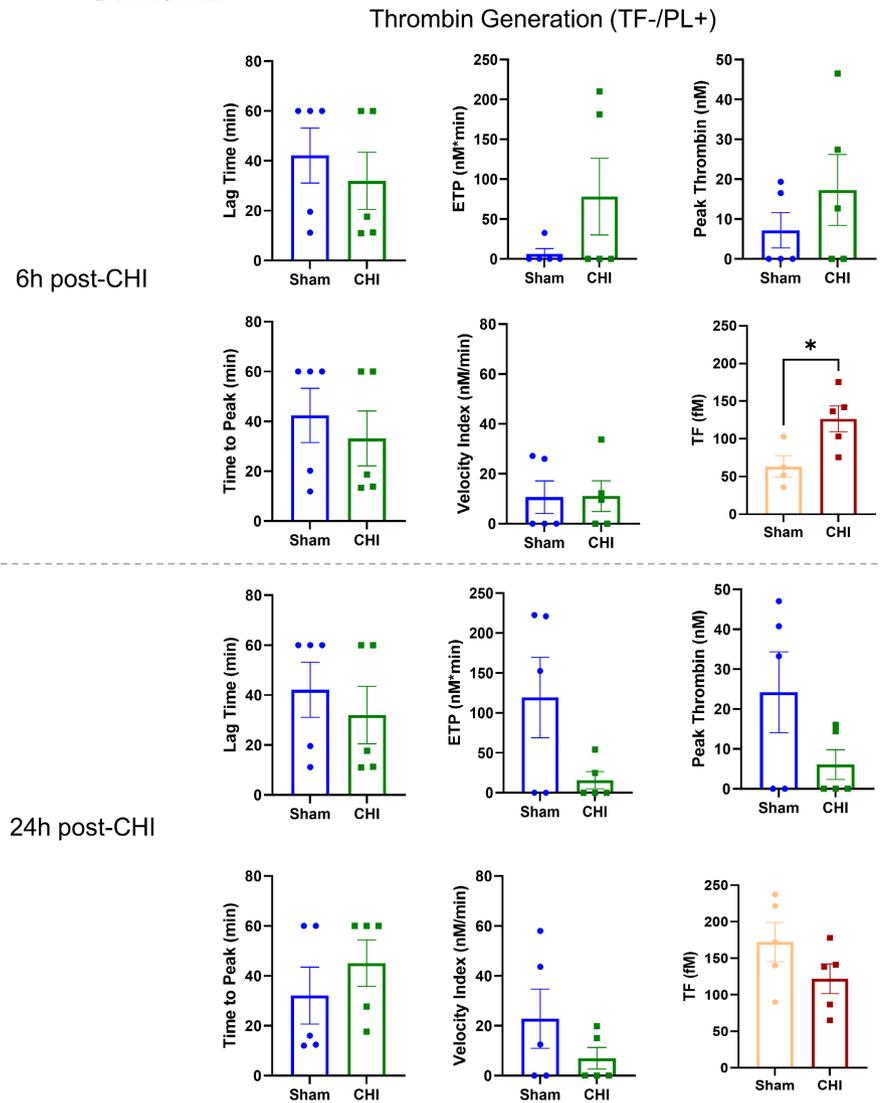
Thrombin Generation (TF-/PL+)

FIGURE 4 Early increase in TF is observed in a noncraniotomy model of mild TBI. Mice received either sham injury or mild CHI followed by euthanasia at either 6 or 24 h after injury. PPP was assayed for thrombin generation. Lag time, ETP, peak thrombin, time to peak, and velocity index were calculated under addition of TF−/PL+. TF activity was also measured in the same samples. There is a significant increase in TF in PPP from CHI mice as compared to sham mice at 6 h after injury. There was no difference in thrombin generation or TF between groups at 24 h after CHI. *$p = 0.03$. $t = 2.74$. $n = 5$/group. Mean ± standard error of the mean and data points. Abbreviations: CHI, closed head injury; ETP, endogenous thrombin potential; PL, phospholipid; PPP, platelet-poor plasma; TBI, traumatic brain injury; TF, tissue factor

parameters (Figure 1D), including lag time, time to peak, endogenous thrombin potential (ETP), and peak thrombin. Mann-Whitney tests were used to compare sham and injury groups. For velocity index and TF activity, parametric analyses were performed by unpaired t test.

3 | RESULTS AND DISCUSSION

A major component of thrombosis following brain injury is release of EVs by damaged neurovasculature. EVs can be procoagulant and contribute to TBI-induced coagulopathy, through the expression of either PSer or coagulation proteins, such as TF. Indeed, elevated levels of TF+ and PSer+ brain-derived microvesicles are present in plasma starting at 3 hours up to 24 hours after brain injury.[14,29] Prior research has established the time course of coagulopathy in experimental closed-skull TBI. There is an immediate hypocoagulation phase[30] followed by a period of hypercoagulation.[31] This period of hypercoagulability is the focus of this study, highlighting our selection of 6-hour and 24-hour time points.

To expand on these findings, we used a model of focal brain injury to investigate the role of TF and phospholipids (PLs) in TBI-induced hypercoagulability. We first assessed whether coagulation factors downstream of TF (see graphical abstract) contributed to thrombin generation after TBI. For this experiment, we measured thrombin generation in the presence of excess TF and PL (Figure 2A). Parameters of thrombin generation were calculated as seen in Figure 1D. There were no significant differences between groups in any parameter of thrombin generation at 6 hours or 24 hours after injury. This suggests that CCI-induced hypercoagulability is not mediated primarily by downstream components of TF. We next assessed the contribution of procoagulant PL by measuring thrombin generation in the presence of excess TF, but no additional PL, making the assay dependent on PL present in the plasma samples. Under these conditions, no significant differences were observed between sham and CCI mice (Figure 2B), suggesting that increased PL is not the driving force in thrombin production following brain injury.

We next measured thrombin generation in the presence of excess PL, with no added TF (Figure 3). At both 6 hours and 24 hours after injury, lag time and time to peak were significantly decreased

in plasma from CCI mice compared to sham mice. Further, plasma from CCI mice displayed higher ETP 6 hours after injury and higher ETP and velocity index 24 hours after injury compared to sham mice. Overall, thrombin generation was significantly faster and higher following CCI. To confirm the contribution of TF to this mechanism, we found that plasma TF activity following CCI was increased two-fold at both 6 hours and 24 hours after injury, and this increase was statistically significant at 24 hours after injury, supporting a TF-driven mechanism of hypercoagulability following TBI (see graphical abstract).

One limitation of the CCI model is the requirement of a craniotomy procedure, which can induce local blood flow changes and damage to the superficial vessels underlying the skull. To interrogate changes in TF activity in non-hemorrhagic brain injury, we performed thrombin generation and TF activity assays in a CHI model (Figure 4). CHI does not produce overt hemorrhage but can result in diffuse vascular disruption.[32] There was significantly elevated plasma TF activity at 6 hours following CHI compared to sham, in which mice with >140 fM TF activity produced substantial levels of thrombin (>150 nM*min). No differences were observed 24 hours after CHI, suggesting a time-dependence to TBI, consistent with reports in patients following TBI or extracranial trauma.[11] It should be noted that differences in TF are driven by the variance of sham TF levels over time; TF levels after CHI are similar at 6 hours and 24 hours after injury. Further, we find that TF levels are much higher in CCI plasma compared with CHI plasma (Figures 3 and 4), indicating that TF release from the brain aligns with the extent of tissue damage and vascular disruption.

These results are consistent with our group's observation of early changes in platelet coupling efficiency after CCI, pointing to TBI-induced alteration of platelet metabolism.[25] Thrombin activates platelets and influences platelet metabolism, as shown by Aibibula et al.[33] In addition, TF+BDEVs, which can be derived from neurons and astrocytes in the TF-rich brain, can also activate platelets.[14] EVs from endothelial cells were also elevated after TBI in one clinical study,[10] suggesting a mechanism of TF release from damaged neurovasculature.

A limitation of the current study is that animals were euthanized by CO_2 asphyxiation that may contribute to changes in coagulability; however, these conditions were identical between experimental groups. Additionally, only male mice were used in these experiments. Additional studies are needed to assess sex-specific responses in TF release and thrombin generation in the context of TBI.

Future directions are poised to examine the ongoing and/or chronic nature of thrombin generation in these models. Patients who are admitted to the emergency department can develop coagulopathy over the first several days following brain injury,[7] indicating possible delayed mechanisms. Further, early mechanisms that lead to fibrin production could contribute to long-term fibrin(ogen) deposition after brain injury. In clinical and preclinical studies, BBB disruption after TBI leads to fibrin(ogen) deposition in cortical vessels, leading to gliosis, reduced neuronal density, and worsened neurological performance.[34-37] Therefore, fibrin(ogen) can drive poor outcomes and early targeting of thrombin generation could mitigate these outcomes.

Our data in CCI and CHI models of TBI show that thrombin generation is mediated, at least in part, by increased EV TF, and that the TF increase is transient and dependent on the type or severity of injury. These findings support the clinical hypothesis that TF drives hypercoagulability after brain injury, and suggests that EV TF may be a valuable biomarker for identifying patients most at risk for thrombotic complications of TBI and for guiding therapeutic interventions.

ORCID

W. Brad Hubbard https://orcid.org/0000-0001-7018-0148
Jeremy P. Wood https://orcid.org/0000-0001-5487-852X

TWITTER

W. Brad Hubbard @bradhubbard1
Martha M. S. Sim @Martha_Sim

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