Published in final edited form as:

J Trauma Acute Care Surg. 2020 November ; 89(5): 926–931. doi:10.1097/TA.0000000000002924.

A rat model of orthopedic injury-induced hypercoagulability and fibrinolytic shutdown

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Abstract

Background—Post-injury hypercoagulability occurs in >25% of injured patients, increasing risk of thromboembolic complications despite chemoprophylaxis. However, few clinically relevant animal models of posttraumatic hypercoagulability exist. We aimed to evaluate a rodent model of bilateral hindlimb injury as a preclinical model of post-injury hypercoagulability.

Methods—Forty Wistar rats were anesthetized with isoflurane: twenty underwent bilateral hindlimb fibula fracture, soft tissue and muscular crush injury, and bone homogenate injection intended to mimic the physiological severity of bilateral femur fracture. Twenty sham rats underwent anesthesia only. Terminal citrated blood samples drawn at 0, 6, 12, and 24h (n=5 per timed group) for analysis by native thromboelastography (TEG) in the presence and absence of taurocholic acid (TUCA) to augment fibrinolysis. Plasminogen activator inhibitor 1 (PAI-1) and alpha-2 antiplasmin (A2AP) levels in plasma were assessed via ELISA.

Results—Injured rats became hypercoagulable relative to baseline by 6h based on TEG MA and G ($p<0.005$); sham rats became hypercoagulable to a lesser degree by 24h ($p<0.005$). Compared to sham animals, injured rats were hypercoagulable by MA and G within 6h of injury, remained hypercoagulable by MA and G through at least 24h (all $p<0.01$), and showed impaired fibrinolysis by TUCA LY30 at 12h ($p=0.019$) and native LY30 at 24h ($p=0.045$). In terms of antifibrinolytic

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Author Contributions

Literature search: KTC, MEK

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Data collection: KTC, ACP, BW

Data analysis: KTC, ACP, MEK

Data interpretation: KTC, ACP, FTS, MEK

Writing: KTC, ACP, FTS, MEK

Critical revision: KTC, ACP, FTS, LM, MEK

Conflict of interest statement

Matthew E Kutcher has served as a member of a Scientific Advisory Board for CSL Behring; however, no related products are discussed in the work presented here.

mediators, A2AP was elevated in trauma animals at 24h ($p=0.009$) and PAI-1 was elevated in trauma animals at 6h ($p=0.004$) and 12h ($p<0.001$) when compared to sham.

Conclusions—Orthopedic injury in rodents induced platelet and overall hypercoagulability within 6 hours and fibrinolytic impairment by 12–24h, mimicking post-injury hypercoagulability in injured patients. This rodent model of orthopedic injury may serve as a preclinical testing ground for potential therapies to mitigate hypercoagulability, maintain normal fibrinolysis, and prevent thromboembolic complications.

Level of evidence—Not applicable; animal study.

Keywords

Hypercoagulability; fibrinolysis; thromboelastography; rodent model

BACKGROUND

Injury is a leading cause of death and disability world-wide, and venous thromboembolism (VTE) is a potentially life-threatening complication for patients who survive their initial injury.(1) Hypercoagulability as a physiological risk for VTE has classically been difficult to study as it is not detected by standard laboratory coagulation tests; however, the recent increasing use of thromboelastography (TEG), a viscoelastic measure of clot formation, has detected early hypercoagulability in 85% of injured patients, and correlated this with a 2-fold increase in the risk of VTE during later recovery.(2, 3) Despite standardized prophylaxis, post-injury VTE rates remain as high as 28% in modern series(4); even under the most stringent prophylaxis regimens with protocolized surveillance, rates of VTE exceed 10%.(5) Thus, the development of novel therapies to mitigate trauma-induced hypercoagulability are urgently needed.

In answer to this, several animal models in various species have been used to study coagulation abnormalities after traumatic injury. Many such models include a component of hemorrhagic shock induced via phlebotomy, despite the fact that the vast majority of injured patients do not present with active hemorrhage. In contrast, orthopedic injury is present in over 45% of trauma patients, and is a well-established risk factor for hypercoagulability.(6, 7) A rodent model of bilateral hindlimb fracture and soft tissue crush injury has been previously described which recapitulates remote organ pathophysiology after injury (including acute kidney injury and pulmonary injury) without hemorrhage.(8, 9) Therefore, we aimed to evaluate whether this clinically relevant rodent model of orthopedic injury reproduces the post-injury hypercoagulability identified in human trauma patients.

METHODS

Rodent Orthopedic Trauma Model

Forty male Wistar Hannover rats ($n=5$ for each treatment and time group) were provided from a colony maintained at our institution and used at 10–18 weeks of age. All experimental protocols were approved by the Institutional Animal Care and Use Committee and carried out in accordance with both the “Guide for the Care and Use of Laboratory

Animals” from the National Institutes of Health and the guidelines of the Animal Welfare Act. Animals were housed 2–3 per cage at 22°C in a 12-hour light/dark cycle and allowed free access to food (Envigo 8640) and water. Under 2–4% isoflurane anesthesia, 20 animals were subjected to (1) controlled soft tissue and muscular injury to muscle beds behind both femurs by 30-second crush with a clamp, (2) fibular fracture using a 16-gauge needle, and (3) injection of 1.5 mL of 0.4g/mL bone homogenate to the injury site (Figure 1). Twenty sham animals underwent isoflurane anesthesia alone. Terminal blood samples were drawn via abdominal aortic puncture into 3.2% sodium citrate at baseline, 6, 12, and 24 hours post-procedure.(10)

Thromboelastography

Citrated whole blood was incubated with gentle rocking at room temperature, re-calcified, and run in duplicate on a TEG5000 Thromboelastograph Hemostasis Analyzer (Haemonetics Corp: Braintree, MA) according to manufacturer’s instructions. Parameters analyzed include R time, coagulation time (K), alpha angle, maximal amplitude (MA), G parameter, and lysis percentage 30 minutes after MA (LY30).

Taurocholic Acid Challenge Fibrinolysis Assay

Based on work by Moore et al, taurocholic acid (TUCA) was used to promote fibrinolysis. (11) Per the previously published protocol, lyophilized TUCA (Sigma Aldrich: St. Louis, MO) was reconstituted to 0.125M in normal saline and stored at 4°C. A dose of 10ul of TUCA was mixed exogenously with 400ul of whole blood prior to TEG analysis; this dose was selected to produce a similar baseline clot lysis between 70–90%.(11) Fibrinolysis was quantified by both native and TUCA-augmented lysis index (LY30) and total clot lysis (CL30) at 30min following maximal amplitude.

Enzyme-linked Immunosorbent Assay (ELISA)

Plasma was obtained by centrifugation from whole blood and flash frozen at –80°C for ELISA analysis of the antifibrinolytic proteins plasminogen activator inhibitor 1 (PAI-1) and alpha-2 antiplasmin (A2AP). Assays for detection of plasma levels of PAI-1 and A2AP were purchased from MyBiosource, Inc (San Diego, CA). Plasma samples were run in duplicate according to the manufacturer’s recommendations and the average of the two samples was used for statistical analysis.

Statistical Analysis

All data are presented as mean $\pm$ standard deviation; univariate comparisons were made using Student’s *t*-test for normally distributed data and Wilcoxon rank-sum testing for skewed data. Effects of treatment and time on TEG parameters and anti-fibrinolytic markers were assessed by two-way analysis of variance (ANOVA) with Tukey’s honest significance post hoc testing to identify significant differences at individual time points. Logarithmic transformation was used to normalize right-skewed data for the native LY30 parameter prior to ANOVA. An alpha of 0.05 was considered significant. All data analysis was performed by the authors using Stata version 12 (StataCorp; College Station, TX).

RESULTS

Rats were used at a mean age of 13.4 ± 1.9 weeks of age and weight of 350 ± 24 g. There were no statistical differences in baseline age (13.5 ± 2.1 vs. 13.9 ± 1.6 weeks, $p=0.3$), weight (350 ± 28 vs. 352 ± 19 g, $p=0.6$), or glucose (166 ± 54 vs. 154 ± 22 mg/dL, $p=0.19$) at the time of terminal blood draw between trauma and sham groups.

There were no differences in R-time, K-time, or alpha angle between trauma and sham animals at 0, 6, 12, or 24 hours (all $p>0.7$; Table 1 and Figure 2A–2C). However, orthopedic trauma produced significant hypercoagulability compared to 0-hour baseline by MA ($p=0.005$) within 6 hours and G ($p=0.002$) within 12 hours after injury, which persisted through 24 hours. Sham animals also became hypercoagulable compared to 0-hour baseline by MA ($p=0.003$) and G ($p=0.001$); however, this did not emerge until 24 hours after the sham procedure, and the degree of hypercoagulability was less than that seen in the trauma group (Table 1 and Figure 2D and E). When compared at each individual time point, injured animals were significantly hypercoagulable compared to sham animals immediately based on MA ($p<0.01$) and by 6 hours post-injury based on G ($p<0.01$), and remained hypercoagulable based on both MA and G compared to sham at 12 and 24 hours ($p<0.001$; Table 1 and Figure 2D and E).

Injured animals also showed evidence of impaired fibrinolysis compared to sham at 24 hours by native LY30 ($p=0.045$; Table 1 and Figure 3A) and at 12 hours by TUCA-LY30 ($p=0.019$; Table 1 and Figure 3B). There were no significant changes compared to baseline seen in the sham group. A summary of fold difference in coagulation parameters between trauma and sham groups at each time point is visually presented as a heat map in Figure 4, reflecting early and persistent enzymatic and platelet hypercoagulability as well as later fibrinolytic impairment in trauma versus sham rats.

Finally, circulating levels of antifibrinolytic factors were assessed by ELISA. Orthopedic trauma resulted in significant increases in plasma A2AP in trauma versus sham animals at 24 hours ($p=0.009$; Table 1 and Figure 5A), and in plasma PAI-1 at 6 ($p=0.004$) and 12 hours ($p<0.001$; Table 1 and Figure 5B). There were no significant differences at any time point compared to baseline seen in the sham group.

DISCUSSION

Coagulopathy is present in approximately 25% of seriously injured patients, and its association with increased transfusion requirements, longer ICU and hospital stay, higher incidence of multiorgan failure, and up to 4-fold higher mortality has been well-described. (12, 13) However, the clinical relevance of hypercoagulability is significantly less understood. Using TEG-based criteria, investigators have shown that 25–65% of trauma patients are hypercoagulable on admission, and that approximately 85% of injured patients eventually develop hypercoagulable profiles overall. (2, 14, 15) Importantly, lower extremity deep venous thrombosis (DVT) rates are higher in patients with hypercoagulable TEG profiles and overall venous thromboembolism (DVT and pulmonary embolisms [PE]) remain as high as 28% with adequate prophylaxis and 58% without chemoprophylaxis. (4,

16, 17) Although the clinical relevance of hypercoagulability is profound, the exact timing and biochemical properties of these coagulation abnormalities are not well-characterized. In our study, rats undergoing bilateral hindlimb fracture exhibited post-traumatic hypercoagulability compared to their sham counterparts beginning within minutes of injury and persisting through at least 24 hours, recapitulating trends toward post-injury hypercoagulability in human trauma patients (Figure 2).

In addition to clot formation-based assessment of hypo- and hypercoagulability, TEG further allows for analysis of fibrinolysis. Three fibrinolytic phenotypes have been characterized in humans based on the TEG LY30 parameter: hyperfibrinolysis (LY30 > 3%), physiologic fibrinolysis (LY30 0.81–2.9%), and fibrinolytic shutdown (LY30 0.0–0.8%).(18) Fibrinolytic shutdown is the most common phenotype after traumatic injury, occurring in anywhere from 46–64% of patients within 12 hours after injury.(18–22) Mortality for the three phenotypes shows a U-shaped distribution with the lowest rate in the physiologic group, while patients with hyperfibrinolysis tend to die early of exsanguination, while patients in shutdown die later with multiple organ failure.(18) While impairment in fibrinolysis has been associated with tissue injury in animal models and with orthopedic surgery in human studies, no specific injury pattern or demographic has been clearly identified that predisposes patients to this phenotype, and no known clinical therapies to modulate fibrinolytic state exist.(18, 23) Assessment of fibrinolytic status in rodents is complicated by baseline resistance to tissue plasminogen activator (tPA)-mediated fibrinolysis.(11) Previous work by Moore *et al.* has described the preincubation of whole blood with taurocholic acid, a pro-fibrinolytic agent, prior to standard TEG analysis as a means of quantifying differences in fibrinolysis in rodents.(11, 24) Using TUGA-augmented TEG, we found that injured animals had impaired fibrinolysis compared to sham beginning at 12 hours and continuing through 24 hours. Mechanistically, this correlated with significant injury-associated elevation of PAI-1 at 6–12 hours, in line with human studies showing that PAI-1 levels are 20 times higher in injured patients with fibrinolytic shutdown,(18) although other work in rodent models has described low baseline expression of PAI-1 in plasma.(11) Further, fibrinolytic shutdown in injured humans has been shown to occur in the absence of elevated PAI-1 and A2AP levels, suggesting that other mechanisms may be playing a role in abnormalities in posttraumatic fibrinolysis.(25) Recently there has been an interest in damage associated molecular patterns (DAMPs), specifically cell-free and mitochondrial DNA, which may suggest additional possible mediators of fibrinolytic shutdown worth investigation in this and other systems.(26, 27) Clinically, the availability of a preclinical model of injury-associated fibrinolytic impairment is of great clinical importance: while hyperfibrinolysis can be readily treated with antifibrinolytics such as tranexamic acid, no commercially available medications with an acceptable side effect profile exist for fibrinolytic shutdown, despite its increasingly recognized prevalence.(28)

The specific use of orthopedic injury rather than acute hemorrhage accurately reflects common injury patterns seen at trauma centers, as only 30% of human trauma patients present with evidence of active hemorrhage - and less than 10% with hemorrhagic shock - while nearly half present with some element of orthopedic injury.(7, 29) In addition to reproducing the hypercoagulability and impairment in fibrinolysis that occur after injury, this model is also advantageous because it has been previously shown to reproduce the

remote organ pathology after injury (including acute lung and kidney injury) in lean and obese Zucker rats, physiologically mimicking the inflammatory response seen after bilateral femur fractures while avoiding the need for operative fixation (an ethical requirement with long bone fractures in rodents).(8, 9, 30)

Several limitations to this study exist. Importantly, hypercoagulable TEG profiles were seen within 24 hours even after the sham procedure, highlighting the likelihood that stress, handling, and anesthetic agents may impact coagulation in ways that remain to be further evaluated. Additionally, human females are known to be hypercoagulable compared to males both at baseline and after injury;(31) the use of male rats only in this study clearly points to the importance of further work on assessing the impact of biological sex on coagulation. As is true of all animal models of injury and particularly true of non-human primates, generalizability to human patients must be considered with caution, but provides an important preclinical platform for mechanistic testing.

In conclusion, we present a model of rodent bilateral hindlimb fracture which mimics the dynamics of early hypercoagulability and later fibrinolytic shutdown seen in injured human trauma patients. This may serve as a preclinical model for the assessment of therapies to mitigate hypercoagulability, maintain normal fibrinolysis, and prevent thromboembolic complications.

Acknowledgments

The authors would like to thank Dr. Lusha Xiang for his assistance with the rodent model. Kristen T Carter is supported by NIH T32 HL-105324. Matthew E Kutcher is supported by NIH U54 GM-115428 from the Mississippi Center for Translational Research and NIH UL1 TR-003096 from the University of Alabama at Birmingham Center for Clinical and Translational Science. Frank T Spradley is supported by NIH R00 HL-130577 and P20 GM-121334 from the Mississippi Center of Excellence in Perinatal Research. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Funding Sources

This study was supported by the following NIH grants:

T32 HL-105324 (KTC)

R00 HL-130577 (FTS)

P20 GM-121334 (FTS)

U54 GM-115428 (MEK)

UL1 TR-003096 (MEK)

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Figure 1. Rodent model of bilateral hindlimb orthopedic injury.

Following isoflurane anesthesia: (1) controlled soft tissue and muscular crush injury is performed by clamping the muscle bed behind the femur for 30 seconds using a right-angle clamp. (2) The fibula is then broken using a 16g needle. (3) 1.5 mL of freshly prepared bone homogenate is injected into the muscle bed behind the femur. (4) The procedure is repeated on the opposite side.

Figure 2. Enzymatic and platelet TEG parameters over time in sham versus trauma animals. Longitudinal citrated native thromboelastography parameters in trauma (solid line) vs. sham (dashed line) animals, including (A) R-time, (B) K-time, (C) alpha angle, (D) maximal amplitude, and (E) G value. Error bars = 95% confidence intervals. Statistical differences marked with * between treatment groups at each time point, and # between 0-hour baseline and each time point, based on two-way ANOVA with Tukey post hoc testing.

Figure 3. Fibrinolysis TEG parameters over time in sham versus trauma animals.

Longitudinal citrated native (A) and taurocholic acid-augmented (B) thromboelastography lysis parameters in trauma (solid line) vs. sham (dashed line) animals. CN: citrated native; TUCA: taurocholic acid; LY30: lysis index at 30min after maximal amplitude. Error bars = 95% confidence intervals. Statistical differences marked with * between treatment groups at each time point, and # between 0-hour baseline and each time point, based on two-way ANOVA with Tukey post hoc testing.

Figure 4. Heat map showing fold difference results of TEG parameters over time of trauma animals compared to sham.

Parameters categorized from relatively hypercoagulable (red) to relatively hypocoagulable (blue). Significant values based on t tests and Mann-Whitney U tests are bolded.

Figure 5. Plasma levels of antifibrinolytic mediators.

Longitudinal ELISA-based plasma (A) alpha-2 antiplasmin (A2AP) and (B) plasminogen activator-inhibitor-1 (PAI-1) in trauma (solid line) vs. sham (dashed line) rats. Error bars = 95% confidence intervals. Statistical differences marked with * between treatment groups at each time point, and # between 0-hour baseline and each time point, based on two-way ANOVA with Tukey post hoc testing.

Table 1.
TEG parameters and antifibrinolytic mediators over time in sham versus trauma animals.

Longitudinal citrated native and taurocholic acid (TUCA)-challenged thromboelastography parameters and antifibrinolytic mediator levels by sham vs. trauma treatment group and time point. Data presented as mean $\pm$ standard deviation or median (interquartile range).

	Parameter	Time point			
		0 h	6 h	12 h	24 h
Sham	R-time (min)	7.5 $\pm$ 2.8	7.6 $\pm$ 1.3	7.5 $\pm$ 3.0	5.8 $\pm$ 3.4
	K-time (min)	1.9 $\pm$ 1.3	2.0 $\pm$ 1.0	2.1 $\pm$ 1.8	1.5 $\pm$ 1.0
	Alpha angle (degrees)	67.2 $\pm$ 12.3	66.7 $\pm$ 9.8	65.8 $\pm$ 14.0	72.8 $\pm$ 12.6
	Maximal amplitude (mm)	67.9 $\pm$ 5.6	71.9 $\pm$ 4.2	72.3 $\pm$ 6.0	75.7 $\pm$ 4.5 [#]
	G value ($\times 10^3$)	11.0 $\pm$ 2.6	13.2 $\pm$ 3.0	13.7 $\pm$ 3.4	16.2 $\pm$ 3.5 [#]
	Native LY30 (%)	0 (0 – 0.5)	0 (0 – 0.1)	0 (0 – 1.7)	0.2 (0 – 2.0)
	TUCA LY30 (%)	63.4 $\pm$ 23.4	47.5 $\pm$ 22.8	75.2 $\pm$ 15.7	61.3 $\pm$ 32.7
	Alpha-2 antiplasmin (ng/mL)	0.53 $\pm$ 0.35	0.51 $\pm$ 0.18	0.48 $\pm$ 0.14	0.25 $\pm$ 0.17
	PAI-1 (ng/mL)	0.54 $\pm$ 0.17	0.56 $\pm$ 0.12	0.19 $\pm$ 0.09 [#]	0.33 $\pm$ 0.31
Trauma	R-time (min)	6.7 $\pm$ 2.8	4.7 $\pm$ 2.8	6.6 $\pm$ 4.9	7.0 $\pm$ 3.5
	K-time (min)	1.6 $\pm$ 1.2	1.4 $\pm$ 1.2	1.9 $\pm$ 1.8	1.8 $\pm$ 1.6
	Alpha angle (degrees)	70.5 $\pm$ 10.9	74.9 $\pm$ 13.5	70.3 $\pm$ 16.6	69.6 $\pm$ 16.0
	Maximal amplitude (mm)	72.1 $\pm$ 4.5	77.8 $\pm$ 3.4^{*,#}	79.7 $\pm$ 4.8^{*,#}	84.0 $\pm$ 1.0^{*,#}
	G value ($\times 10^3$)	13.3 $\pm$ 2.6	17.9 $\pm$ 2.9[*]	19.6 $\pm$ 8.0^{*,#}	26.3 $\pm$ 2.0^{*,#}
	Native LY30 (%)	0 (0 – 0.1)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0) [*]
	TUCA LY30 (%)	68.1 $\pm$ 22.5	46.0 $\pm$ 33.5	41.9 $\pm$ 32.6[*]	56.2 $\pm$ 25.0
	Alpha-2 antiplasmin (ng/mL)	0.46 $\pm$ 0.27	0.40 $\pm$ 0.23	0.56 $\pm$ 0.20	0.63 $\pm$ 0.30[*]
	PAI-1 (ng/mL)	0.39 $\pm$ 0.16	1.06 $\pm$ 0.45^{*,#}	0.49 $\pm$ 0.19[*]	0.22 $\pm$ 0.14

* Statistical differences marked with between treatment groups at each time point

between 0-hour baseline and each time point within treatment group, based on two-way ANOVA with Tukey post hoc test.