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## Shock Releases Bile Acid Inducing Platelet Inhibition and Fibrinolysis

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### Abstract

**Introduction**—Metabolites are underappreciated for their effect on coagulation. Taurocholic acid (TUCA), a bile acid, has been shown to regulate cellular activity and promote fibrin sealant degradation. We hypothesize that TUCA impairs whole blood clot formation and promotes fibrinolysis.

**Methods**—TUCA was exogenously added to whole blood obtained from volunteers. A titration from 250  $\mu$ M to 750  $\mu$ M was utilized due to biologic relevance. Whole blood mixtures were assayed using thrombelastography (TEG) for clot strength (MA) and fibrinolysis (LY30) quantification. Tranexamic acid (TXA) was used to block plasmin mediated fibrinolysis. Platelet microfluidics were performed. A proteomic analysis was completed on citrated plasma obtained from a shock and resuscitation rat model.

**Results**—Fibrinolysis increased, when 750  $\mu$ M TUCA was added to whole blood (median LY30 0.08 to 5.7,  $p=0.010$ ), and clot strength decreased (median MA of 53.3 to 43.8,  $p=0.010$ ). The addition of TXA, to a 750  $\mu$ M TUCA titration, partially reversed the induced fibrinolysis (LY30: without 7.7 vs. with 2.7) and the decrease in clot strength (MA: without 48.2 vs. with 53.2), but

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did not reverse the effects to whole blood levels. Platelet function reduced by 50% in the presence of 100  $\mu$ M TUCA. Rats had a median 52-fold increase in TUCA, following a shock state that stayed elevated following resuscitation.

**Conclusion**—TUCA reduces clot strength and promotes fibrinolysis. The clot strength reduction is attributable to platelet inhibition. This metabolic effect on coagulation warrants further investigation, as localized areas of the body, with high levels of bile acid, may be at risk for postoperative bleeding.

## Keywords

taurocholic acid; bile salt; thrombelastography; fibrinolysis; clot strength; platelet dysfunction

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## 1. Introduction

Bile salts are unique amphipathic molecules that have been found to enhance tissue plasminogen activator (tPA) mediated fibrinolysis [1]. Normal levels of bile acids in systemic circulation are on the micromolar level [2]. However, bile acids such as taurocholic acid (TUCA) have been found to be elevated by 1000 fold during pathologic conditions [3]. In vitro TUCA causes angiogenesis and cell proliferation [4]. Elevated blood TUCA levels have also been implicated with heart arrhythmias [5], suggesting that this is a highly active biologic molecule. In rat models, it has been shown that serum taurocholic acid levels markedly rise following acute liver injury [6,7].

Previous studies evaluating the effect of TUCA on coagulation have not used whole blood assays. Hoffman and Monroe [8] have elucidated the cell based model of hemostasis, emphasizing the key role of platelets. Because of the many receptors on platelets and biologic activity of TUCA, we hypothesize that this bile acid impairs whole blood clot formation via platelet inhibition, that it promotes fibrinolysis, and that this metabolite can be released following hemorrhagic shock due to oxygen sensitivity of the liver.

## 2. Methods

### 2.1 Subjects

After obtaining informed consent under an institution review board approved protocol (COMIRB # 14-0366) blood samples were obtained from healthy volunteers with no known abnormalities in the coagulation or fibrinolytic system; none of these individuals were taking salicylic acid or other non-steroidal anti-inflammatory medications within 120 hours of the experiment. All subjects were male with a median age of 28 years, range 20–37.

### 2.2 Taurocholic Acid (TUCA)

Lyophilized taurocholic acid, purchased from sigma Aldrich (Product # 86993, St. Louis, MO), was reconstituted to 0.125 M in normal saline. Aliquots of 40  $\mu$ L of this solution were stored at 4 °C until use.

### 2.3 Thrombelastography TUCA Challenge (TEG TUCA Challenge)

Blood was collected in 3.3ml citrated blood tubes from venous puncture. Citrated samples were kept at room temperature and assayed between 20 minutes and 2 hours after blood draw, in accordance with manufacturer recommendations. Whole blood was mixed with TUCA to reach a final volume of 500 microliters in individual Eppendorf tubes (Hamburg, Germany). The concentration of TUCA ranged from 0–750 micromolar ( $\mu\text{M}$ ). TUCA was added to whole blood mixtures within 5 minutes of initiating the assay. Citrated native thrombelastography (TEG) assays were re-calcified and run according to the manufacturer's instructions on a TEG 5000 Thrombelastograph Hemostasis Analyzer (Haemonetics, Niles IL). The following parameters were recorded from the tracings of the TEG: R time (seconds), angle ( $\alpha$ , degrees), maximum amplitude (MA, mm), and lysis 30 minutes after MA (LY30, %). To detect the presence of plasmin-mediated fibrinolysis, tranexamic acid (TXA) at a concentration of 20mg/ml was mixed with samples.

### 2.4 Platelet Function Assay

A previous validated PDMS flow device [9] was used to create eight evenly spaced flow channels (250  $\mu\text{m}$  in width, 60  $\mu\text{m}$  in height) that are perpendicularly deposited over pre-patterned collagen matrix (250  $\mu\text{m}$  in width). PPACK (irreversible thrombin inhibitor, 100  $\mu\text{M}$ ) treated (+/- taurocholic acid) whole blood was perfused over collagen fibrillar at venous shear rate (100  $\text{s}^{-1}$ ). Dynamic change of platelet (labeled with anti-CD 61 Ab) deposition on collagen was monitored with fluorescence microscopy (Olympus IX81).

### 2.5 Animal Shock Resuscitation Model

Sprague Dawley rats were subjected to hemorrhagic shock (IACUC # 90814), and citrated plasma samples were collected for proteomic and metabolic analysis. In brief, rats were anesthetized with pentobarbital and underwent tracheostomy and femoral artery cannulation. Blood was drawn through the femoral artery. After baseline blood was obtained, animals were hemorrhaged to a mean arterial pressure (MAP) of 25 mm hg for 30 minutes, followed by an additional blood draw. Animals were then resuscitated with normal saline, and blood samples were obtained 15 minutes after animals obtained a MAP greater than 30 mm hg. The volume of resuscitation was limited to less than 20%EBV. A post hoc analysis to assess TUCA was performed on previously run samples corresponding to baseline, shock, and reperfusion plasma.

### 2.6 TUCA Quantification Via Mass Spectroscopy

Plasma samples (10  $\mu\text{l}$ ) were immediately extracted in ice-cold lysis/extraction buffer (methanol:acetonitrile:water 5:3:2) at 1:25 dilutions. Samples were then agitated at 4°C for 30 min and centrifuged at 10,000g for 15min at 4°C. Protein and lipid pellets were discarded, while supernatants were stored at -80°C prior to metabolomics analyses. Ten  $\mu\text{l}$  of sample extracts were injected into an UPLC system (Ultimate 3000, Thermo, San Jose, CA, USA) and run on a Kinetex XB-C18 column (150×2.1 mm i.d., 1.7  $\mu\text{m}$  particle size – Phenomenex, Torrance, CA, USA) using a 9 min gradient at 250  $\mu\text{l}/\text{min}$  (mobile phase: 5% acetonitrile, 95% 18 m $\Omega$  H<sub>2</sub>O, 0.1% formic acid). The UPLC system was coupled online with a QExactive system (Thermo, San Jose, CA, USA), scanning in Full MS mode (2

µscans) at 70,000 resolution in the 60–900 m/z range, 4kV spray voltage, 15 sheath gas and 5 auxiliary gas, operated in negative and then positive ion mode (separate runs). Calibration was performed before each analysis against positive or negative ion mode calibration mixes (Piercenet – Thermo Fisher, Rockford, IL, USA) to ensure sub ppm error of the intact mass. Taurocholate assignments was performed using the software Maven [10] (Princeton, NJ, USA), upon conversion of .raw files into .mzXML format through MassMatrix (Cleveland, OH, USA). The software allows for peak picking, feature detection, and metabolite assignment against the KEGG pathway database. TUCA assignment was further confirmed against chemical formula determination (as gleaned from isotopic patterns and sub 1 ppm error on accurate intact mass), and retention times were confirmed against commercially available hydrophobic standard libraries (SIGMA Aldrich, St. Louis, MO, USA; IROATech, Bolton, MA, USA).

## 2.7 Statistical Analysis

SPSS software version 22 (IBM, Armonk, NY) was used for statistical analysis. The TEG value LY30 and MA in addition to TUCA concentrations had a skewed, non-normal distribution. Spearman's Rho was used to assess the correlation of TEG values and concentration of TUCA in whole blood (0, 250, 500, 750 µM). Comparisons between groups LY30 with and with out TUCA and TXA were performed with the non-parametric paired Friedman's test with the stepwise adjustment for multiple comparisons. TUCA plasma levels in rat from baseline, shock, and reperfusion were contrasted with the same statistical methodology. Platelet fluorescent intensity in microfluidic assay was averaged across three clotting events ( $\pm$  SD, shaded area). Significance was set at two-tailed alpha of 0.05 for all statistical tests.

## 3. Results

### 3.1 Increasing Whole Blood Concentration of TUCA correlates with LY30 and MA

Whole blood median LY30 was 0.08% (IQR 0–1.35). Increasing the molar concentration of TUCA correlated with an increase in LY30 (Spearman's Rho = 0.743  $p < 0.001$ ). LY30 was statistically increased when TUCA concentration was 750 µM (5.7 IQR 5.0–7.7) compared to baseline blood ( $p = 0.010$  figure 1). Clot strength also showed a relationship to TUCA concentration. Whole blood median MA was 53.3 (IQR 49.5–58.5). Increasing the concentration of TUCA correlated with a decrease in MA (Spearman's Rho =  $-0.479$   $p = 0.018$  figure 2). Clot strength was significantly reduced at TUCA concentration of 750µM (MA = 43.8 IQR 41.5–48.3) compared to baseline ( $p = 0.010$ ). Other TEG parameters (R time Spearman's Rho =  $-0.098$   $p = 0.613$  and Angle Spearman's Rho =  $-0.135$   $p = 0.485$ ) did not have a correlation with TUCA concentration.

### 3.2 Tranexamic Acid Partially Reverses LY30 and MA Changes Associated With TUCA

The addition of TXA to whole blood did not change LY30 (without 1.6 IQR 0–2 vs. with 1.35 IQR 0.1–1.7  $p = 0.343$ ) or MA (without 56.5 IQR 53.4–58.6 vs. with 56.3 IQR 52.9–57.4  $p = 0.408$  figure 3). Reversal of the effect of TUCA at 750µM was significant with TXA for LY30 (without 7.7 IQR 5.7–14.2 vs. with 2.7 IQR 1.85–3.3  $p = 0.028$ ) and MA (without 48.2 IQR 43.8–52.4 vs. with 53.2 IQR 48–56.1  $p = 0.048$ ). However, LY30 remained

significantly higher in the blood with 750  $\mu\text{M}$  concentration TUCA with TXA compared to whole blood ( $p=0.028$ ) in addition to having a lower MA ( $p=0.048$ ).

### 3.3 TUCA Inhibits Platelet Function

Platelet signal in the initiation phase was identical in control and treated group. Signal of treated group started to deviate from control after the first 2 minutes indicating possible effect of taurocholic acid on platelet secondary aggregation. This effect should be independent of thrombin since thrombin was depleted by PPACK. The reduction in platelet signal with 100  $\mu\text{M}$  TUCA reached 50% by the end of the assay suggesting taurocholic acid is a potent antiplatelet agent (figure 4).

### 3.4 TUCA Concentrations Increase Following Hemorrhagic Shock and Early Resuscitation

Rats' baseline TUCA was 10,500 AU (IQR 0–31,500). After shock all rats had an increase in median TUCA plasma level to 550,000 AU (IQR 67,000–1,237,500). After resuscitation TUCA levels remained elevated (median 525,500 AU IQR 142,500 – 1,725,000). Pairwise adjustment of groups based on TUCA levels from baseline trended towards significance for shock ( $p=0.063$ ) and was significant after resuscitation ( $p=0.012$  figure 5).

## 4. Discussion

Taurocholic acid is a potent liver metabolite that impairs platelet function and promotes fibrinolysis. In vitro assays have identified a dose like effect in which increasing the concentration of TUCA increases LY30 and reduces MA. Tranexamic acid can partially reverse these effects, but is not capable of completely blocking fibrinolysis and reduction of clot strength. In the absence of fibrin generation, TUCA impairs platelet aggregation by up to 50%, at a much lower concentration than required to impair clotting parameters detected by TEG in whole blood. An animal model of shock and resuscitation supports that TUCA levels can increase within 30 minutes of low blood pressure and remain elevated after resuscitation.

Bile acids have previously been associated with platelet dysfunction [11]. The proposed mechanism was through membrane deformity. Phospholipid membrane on platelets has been implicated as critical for these cells to aggregate and degranulate [12]. However bile acids also activate a number of cellular calcium related channel receptors [5,13,14]. Calcium regulation is critical for multiple platelet functions [15], and may be another mechanism for TUCA inhibition of platelets. We have shown that platelet aggregation is inhibited by TUCA using microfluidics (figure 4). We have also shown that clot strength can be reduced as measured by thrombelastography (figure 2), which is perceived to reflect platelet function [16].

Tranexamic acid partially reverses the TUCA reduction in clot strength, and suggests a potential receptor-mediated mechanism. TXA is perceived to be an antifibrinolytic agent, but is known to interact with multiple receptors [17]. TXA has recently been suggested to improve platelet function in patients on dual antiplatelet therapy [18], but the mechanism remains unknown. The reduction of LY30 with TXA in the presence of TUCA does support some degree of a plasmin-mediated process. Boonstra et al. had previously shown that

TUCA could suppress PAI-1 activity allowing for uninhibited tissue plasminogen activation of plasminogen causing fibrinolysis [1]. The receptor-mediated effects of TUCA warrant further investigation, as it may involve previously unrecognized receptors and pathways.

In multiple rat studies, acute liver injury increased systemic TUCA levels above control levels [6,7]. In our animal study, plasma from 30 minutes of systemic shock also contained increased TUCA levels. These animal models suggest that both an ischemic insult and mechanical damage to the liver can release TUCA locally and systemically. This correlates to clinical outcomes in which the volume of liver removed (tissue injury)[19,20], pringle time (local ischemia)[19,21], and blood loss (systemic ischemia)[22] are associated with an increased risk of post-operative complications. While TUCA levels have not been clinically measured at the cut edge of a liver, the concentration is likely to be markedly elevated. The gastric contents from patients with pathologic reflux have been measured to have TUCA concentrations 1000 times greater than plasma levels [3]. This is likely is a lower level estimate of TUCA concentrations from intrahepatic bile which is not diluted by gastric contents. However, TXA does partially suppress both the loss of clot strength and increase in fibrinolysis, therefore it may be a potential topical therapeutic for difficult to control liver bleeding.

This study is limited to an in vitro analysis on healthy volunteer blood. Because of the testing environment, the blood contained normal levels of all liver synthesized proteins and blood components, which is not the case for many trauma patients. Additionally, the testing environment only looked at the interaction of the one metabolite. There can be other metabolite interactions that are not being assessed during our study, as well as cellular and organ specific effects. TUCA has previously been implicated in causing impairment of fibrinolysis in a rat acute pancreatitis model [23]. However these effects were not appreciated until 3 hours after injection with evidence of inflammation of the pancreas and systemic levels of TUCA were not analyzed. This observation may have been an adaptive response to inflammation, which has previously been shown to shutdown fibrinolysis [24]. It would not be unexpected that TUCA has tissue dependent effects on fibrinolysis.

In conclusion, our results have shown that taurocholic acid has the ability to cause platelet dysfunction, in addition to increased fibrinolysis. TUCA has the potential to create post-operative complications in trauma and liver patients by impaired hemostasis and containment of bile, in addition to causing systemic impairment of coagulation. With the use of rapid output metabolomics our understanding of the effect of many previously unmeasured small biologic molecules will likely change our understanding of the bodies response to surgical procedures and shock. TUCA provides an example of a small highly active biological molecule that previously received minimal attention on its impact on coagulation.

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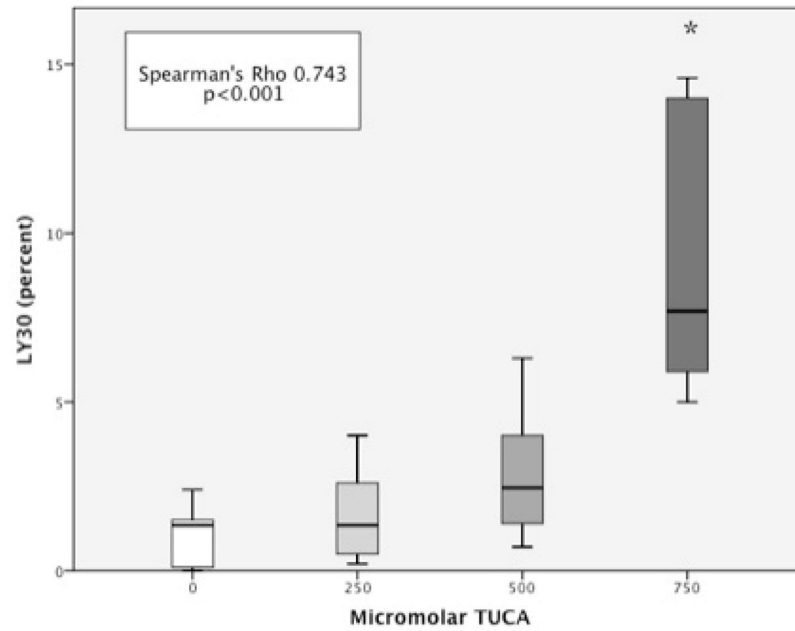


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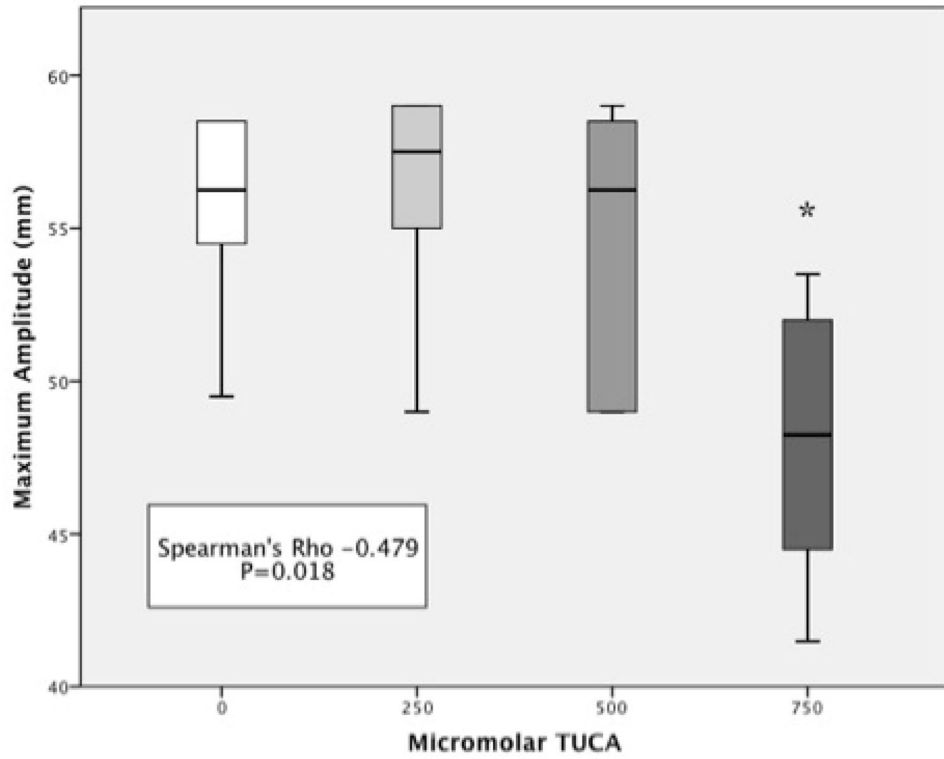
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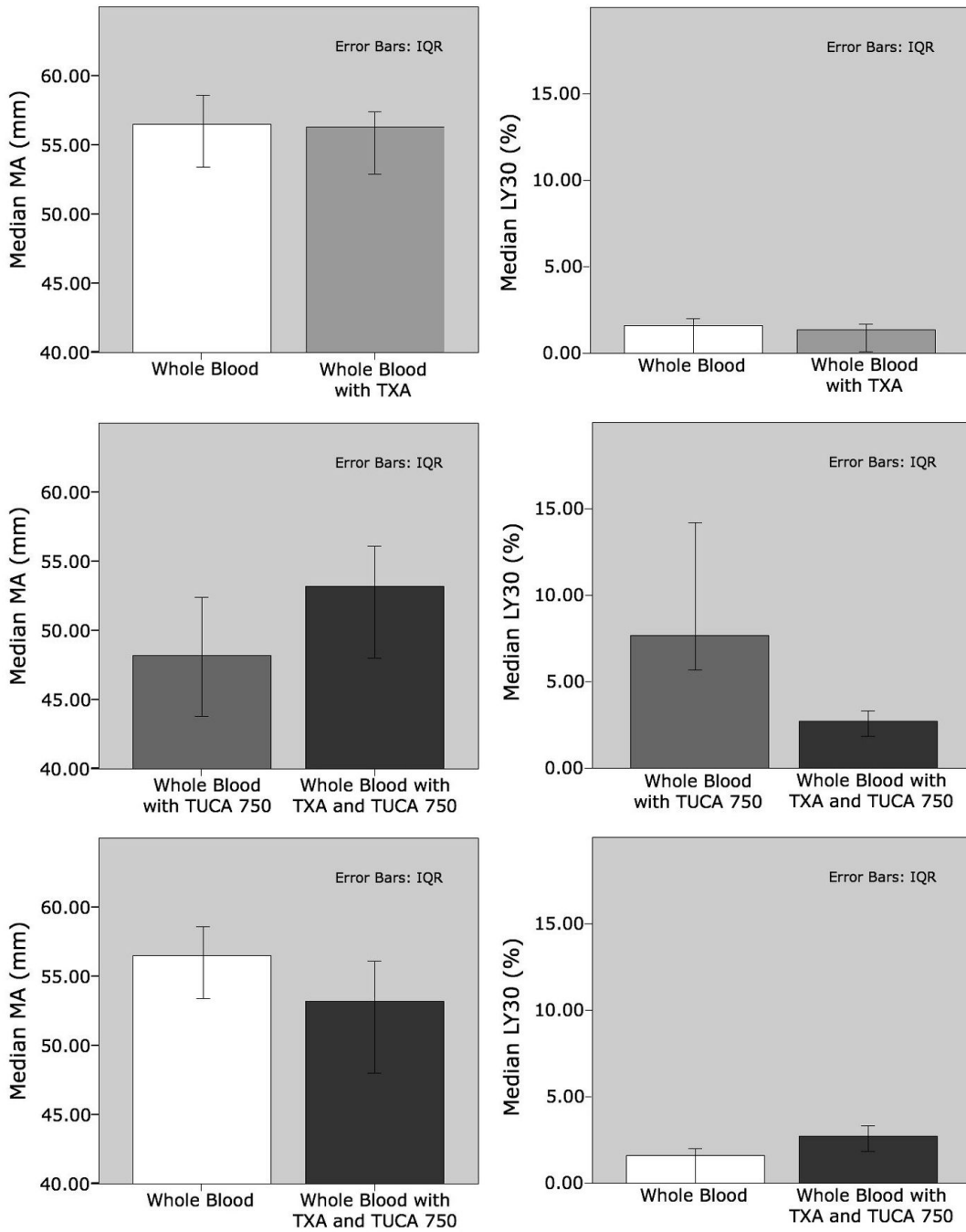
**Figure 1. Fibrinolysis Increases With Increasing Concentration of TUCA**

\* =  $P < 0.05$ , LY30 = Percent fibrinolysis 30 minutes after maximum amplitude, TUCA = Taurocholic Acid. Y-axis represents the percent of lysis 30 minutes after blood clot reaches maximum amplitude. The X-axis represents a progressive concentration of TUCA in whole blood ranging from 0 (left) to 750 micromolar (right).



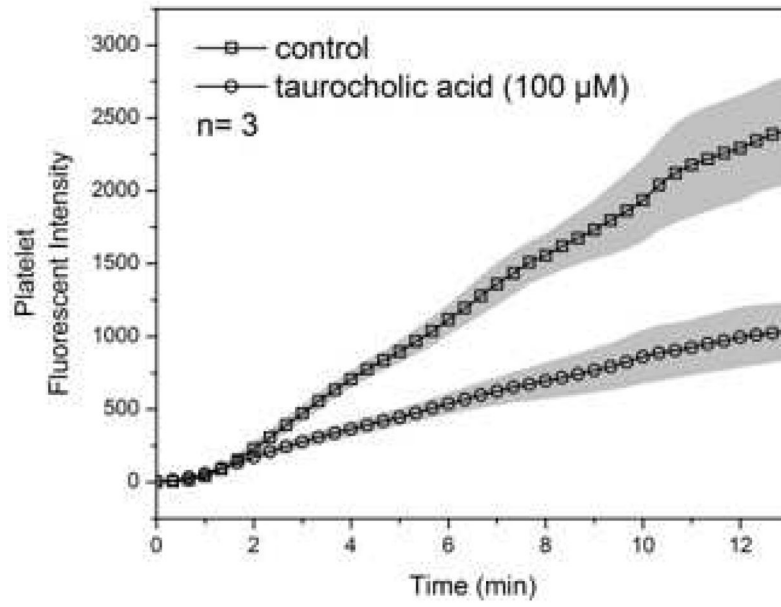
**Figure 2. Clot Strength Decreases With Increasing Concentration of TUCA**

\* =  $P < 0.05$ , MA = Maximum amplitude of clot formation, TUCA = Taurocholic Acid. Y-axis represents the maximum amplitude of formed blood clot. X-axis represents a progressive concentration of TUCA in whole blood ranging from 0 (left) to 750 micromolar (right).



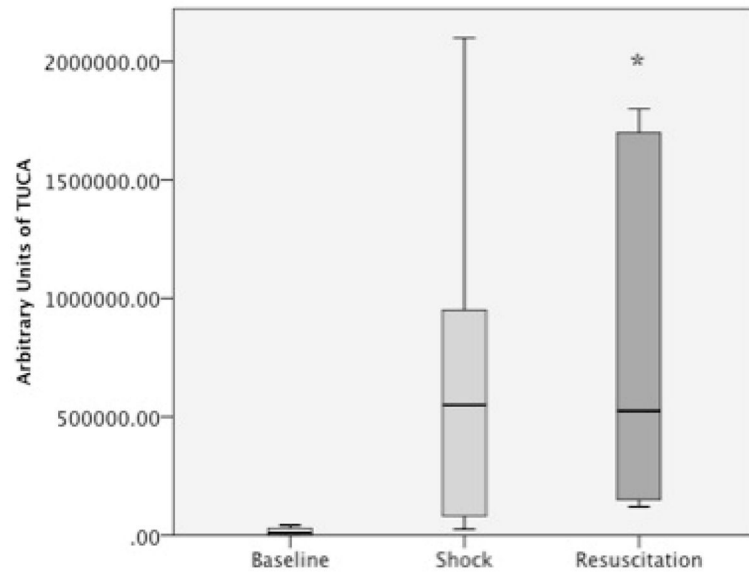
**Figure 3. Tranexamic Acid Only Partially Reverses the Affects of Taurocholic Acid on Whole Blood Clot Formation**

LY30 = Percent fibrinolysis 30 minutes after maximum amplitude, MA = Maximum amplitude of clot formation, TUCA = Taurocholic Acid, TXA = Tranexamic Acid. Row 1 demonstrates MA and LY30, for whole blood without TUCA, without and with TXA. Row 2 demonstrates MA and LY30 for whole blood with TUCA, without and with TXA. Row 3 correlates MA and LY30 for whole blood without TUCA and TXA verses whole blood with TUCA and TXA.



**Figure 4. Taurocholic Acid Inhibits Platelet Function**

Y-axis shows the signal production relating to platelet activity. X-axis shows the elapsed time.



**Figure 5. Taurocholic Acid Increases in Rats Following Shock and Stays Elevated During Resuscitation**  
 TUCA = Taurocholic Acid. Y-axis represents arbitrary quantitative units of taurocholic acid. X-axis represents the 3 stage points for blood analysis during the model.

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