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ORIGINAL ARTICLE

Robust coagulation activation and coagulopathy in mice with experimental acetaminophen-induced liver failure

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Abstract

Background: Patients with acetaminophen (APAP)-induced acute liver failure (ALF) display both hyper- and hypocoagulable changes not necessarily recapitulated by standard hepatotoxic doses of APAP used in mice (eg, 300 mg/kg).

Objectives: We sought to examine coagulation activation *in vivo* and plasma coagulation potential *ex vivo* in experimental settings of APAP-induced hepatotoxicity and repair (300-450 mg/kg) and APAP-induced ALF (600 mg/kg) in mice.

Results: APAP-induced ALF was associated with increased plasma thrombin–antithrombin complexes, decreased plasma prothrombin, and a dramatic reduction in plasma fibrinogen compared with lower APAP doses. Hepatic fibrin(ogen) deposits increased independent of APAP dose, whereas plasma fibrin(ogen) degradation products markedly increased in mice with experimental ALF. Early pharmacologic anticoagulation (+2 hours after 600 mg/kg APAP) limited coagulation activation and reduced hepatic necrosis. The marked coagulation activation evident in mice with APAP-induced ALF was associated with a coagulopathy detectable *ex vivo* in plasma. Specifically, prolongation of the prothrombin time and inhibition of tissue factor–initiated clot formation were evident even after restoration of physiological fibrinogen concentrations. Plasma endogenous thrombin potential was similarly reduced at all APAP doses. Interestingly, in the presence of ample fibrinogen, ~10 times more thrombin was required to clot plasma from mice with APAP-induced ALF compared with plasma from mice with simple hepatotoxicity.

Conclusion: The results indicate that robust pathologic coagulation cascade activation *in vivo* and suppressed coagulation *ex vivo* are evident in mice with APAP-induced ALF. This unique experimental setting may fill an unmet need as a model to uncover mechanistic aspects of the complex coagulopathy of ALF.

KEYWORDS
acute liver failure, coagulation, coagulopathy, mouse model

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1 | INTRODUCTION

Acetaminophen (paracetamol, APAP) overdose is a leading cause of drug-induced acute liver injury (ALI) and acute liver failure (ALF) in the United States and other developed countries [1]. Both APAP-induced ALI and ALF are associated with coagulopathy, characterized by an increased prothrombin time (PT) and international normalized ratio (INR) [2]. Reduced concentrations of procoagulant factors such as prothrombin, factor V, factor VII, and fibrinogen, which contribute to the increased INR, are observed in ALI and ALF patients [3,4]. Reduction in plasma concentrations of the natural anticoagulants protein C, protein S, and antithrombin also occurs in ALI/ALF patients [3,4]. The net effect of these changes is development of a complex “rebalanced” hemostatic system [4]. Abnormalities in blood clot formation detected by rotational thromboelastometry (ROTEM) suggest hypocoagulability in patients with ALF [5]. In contrast, hypercoagulable thromboelastography (TEG) features have also been observed in patients with ALF [6]. Likewise, increased severity of hepatic encephalopathy, a hallmark of ALF, was associated with an increase in endogenous thrombin potential [7]. These studies illustrate the challenge in defining the precise changes in coagulation evident in patients with ALI or ALF. Notably, the mechanism(s) driving the complex changes in coagulation in ALI and ALF is not fully understood.

Many fundamental mechanisms of APAP hepatotoxicity have been elucidated using a reproducible mouse model in which mice are challenged with a single dose of APAP (eg, 300 mg/kg). Mice challenged with 300 mg/kg APAP developed substantial liver injury (peak ∼18-24 hours), followed by resolution of injury and tissue repair within approximately 72 hours after APAP challenge [8]. Using this approach, we previously showed that the coagulation system is rapidly activated and causes deposition of hemostatic components such as fibrin(ogen), platelets, and von Willebrand factor in the injured liver [9–11]. Components of the hemostatic system play a complex role in the progression of liver injury and repair after standard APAP overdose in mice [10,12–14]. However, whereas liver repair prevents severe hepatic dysfunction in mice challenged with 300 mg/kg APAP, larger doses of APAP (500-600 mg/kg) produce extensive liver injury with substantial inhibition of liver repair and regeneration, delaying recovery and reducing survival [15]. Indeed, indices of hepatic encephalopathy emerge in mice challenged with these larger doses of APAP [16,17].

Although the contribution of the hemostatic system to liver injury and repair after standard APAP overdose has been extensively studied [9–11,13,14,18–22], the precise changes and role of coagulation in experimental APAP overdose more closely resembling ALF have not been examined. We posited that the exploration of changes in coagulation observed across the APAP dose–response could help reveal the basis for the complex changes in coagulation evident in patients with acute liver injury. We therefore sought to define the hemostatic abnormalities in the established experimental settings of APAP hepatotoxicity and ALF-like conditions produced by larger doses of APAP (ie, 600 mg/kg).

Essentials

- Acute liver injury in experimental settings induces activation of the coagulation cascade.
- Acetaminophen (APAP) induces dose-dependent liver injury and acute liver failure in mice.
- High-dose APAP (600 mg/kg) causes severe coagulopathy compared with standard hepatotoxic doses.
- Mice with APAP-induced acute liver failure display reduced *ex vivo* thrombin-mediated plasma clotting.

2 | METHODS

2.1 | Mice

Male C57Bl/6J wild-type mice (Jackson Laboratories) between the ages of 8 and 10 weeks were used for these studies. Mice were housed under a 12-hour light/dark cycle, and standard diet and drinking water were provided *ad libitum*. The Institutional Animal Care and Use Committee of Michigan State University approved all animal procedures.

2.2 | APAP-induced ALI and ALF and determination of liver injury

Mice were fasted overnight before administration of 300, 450, or 600 mg/kg APAP (60 μL/g body weight) or vehicle (sterile saline, 0.9% sodium chloride) via intraperitoneal (i.p.) injection. Two independent studies were performed, and where possible, biomarkers from each study were measured simultaneously. These doses were selected based on prior studies using male mice in which doses causing acute hepatotoxicity and failed liver repair were identified [15]. To inhibit thrombin, mice were treated 2 and 12 hours after APAP challenge with dabigatran etexilate (DE; 10 mg/kg, i.p.; Cayman Chemical) or vehicle (2% dimethyl sulfoxide, 30% glycerinformal-cremophor). Liver, kidney, and plasma samples were collected 24 hours after APAP challenge. Liver necrosis was assessed in hematoxylin and eosin (H&E)-stained liver sections, as described previously [23]. Kidneys were examined by a board-certified veterinary pathologist (VW) for lesions of acute kidney injury, such as tubular degeneration, hyaline and cellular casts, and necrosis. Plasma alanine aminotransferase (ALT) activity, direct bilirubin, and albumin were determined using commercial reagents (ThermoFisher Scientific and Pointe Scientific). For full details of these methods, see Supplementary Methods.

2.3 | Measurement of hemostatic parameters in plasma

Blood was collected under isoflurane anesthesia by exsanguination from the inferior vena cava immediately after injection of 3.8% sodium

citrate diluted in saline in the spleen as previously described [24]. Blood samples were centrifuged at 4000 × *g* for 10 minutes to obtain plasma and were stored at −80°C. Plasma thrombin–antithrombin (TAT) complexes, plasma D-dimer concentrations, and PT were determined using commercially available kits (Siemens Health Care Diagnostics; Diagnostica Stago; Pacific Hemostasis/ThermoFisher Scientific; and Molecular Innovations). Thrombin generation was measured by calibrated automated thrombography, as described [25,26]. Plasma prothrombin concentrations were measured using a commercial ELISA according to the manufacturer's instructions (Molecular Innovations). Plasma fibrinogen concentrations were determined using an in-house ELISA. For full details of these methods, see Supplementary Methods.

2.4 | Detection of hepatic fibrin(ogen) by capillary western blotting

Fibrin(ogen) concentrations were measured in enriched urea-insoluble (ie, cross-linked) tissue extracts using automated capillary western blotting (Wes; ProteinSimple), as described previously [14]. For full details, see Supplementary Methods.

2.5 | Assessment of plasma clot formation by turbidity

Fibrin polymerization was studied by monitoring the changes in turbidity during clot formation initiated with tissue factor (TF, Thromboplastin DS, Pacific Hemostasis) or bovine thrombin (Enzyme Research Laboratories). To initiate with TF, 140 μL of 4× diluted plasma (in 0.1 M Tris–HCl (pH 7.4)) and 35 μL of Thromboplastin DS solution (reconstituted using the manufacturer's instruction, then 2000× diluted in 0.1 M Tris–HCl (pH 7.4), final concentration 12 000×) was added to each well of a 96-well plate. Clotting was initiated by adding 35 μL of 30 mM $CaCl_2$ (in 0.1M Tris–HCl, final concentration 5 mM). To initiate with thrombin, 160 μL of 4× diluted plasma (in 0.1 M Tris–HCl [pH 7.4]) was added to each well of a 96-well plate. Clotting was initiated by adding a 40 μL solution containing 25 mM $CaCl_2$ (final concentration 5 mM) and 1.25, 12.5, or 50 U/mL bovine thrombin (final concentration 0.25, 2.5, or 10 U/mL thrombin, respectively). In some experiments, plasma was supplemented with human fibrinogen (plasminogen depleted, Enzyme Research Labs), final concentration 150 or 200 mg/dL, as indicated. Turbidity curves were generated by monitoring changes in absorbance at 405 nm every 25 seconds for 35 minutes at 37 °C in a microplate reader (Infinite M200 Tecan).

2.6 | Statistics

Statistical analyses were performed using GraphPad Prism v.9 software. Continuous variables are presented as values for individual mice and as mean ± SEM. Comparison of 2 groups was performed using Student's *t*-test. Comparison of 3 or more groups was performed using 1- or 2-way analysis of variance (ANOVA), as appropriate, with the Tukey post hoc test. Differences were considered significant if the *p*-value was $< .05$.

3 RESULTS

3.1 | APAP overdose induces dose-dependent effects on liver function and injury

Plasma ALT (Figure 1A) and direct bilirubin (Figure 1B) increased in a dose-dependent manner. The plasma albumin concentration was significantly decreased only in mice challenged with 600 mg/kg APAP (Figure 1C). Liver injury in APAP-challenged mice was characterized by centrilobular necrosis (Figure 1D, E, black asterisk). Mice challenged with 600 mg/kg APAP had a significantly increased area of hepatocellular necrosis compared with 300 mg/kg APAP-challenged mice (Figure 1D). Moreover, overt hemorrhage/congestion was evident in the livers of mice challenged with 600 mg/kg APAP but not at lower APAP doses (Figure 1E, arrowheads). Swelling of hepatocytes with vacuolation of the cytoplasm was frequently observed in hepatocytes outside distinct areas of necrosis at both the 450 and 600 mg/kg doses (Figure 1F, white triangle). Furthermore, hepatocellular ballooning (Figure 1F, white arrow), as well as sinusoidal perturbation (Figure 1F, black arrow) distinguished mice challenged with 600 mg/kg APAP from lower APAP doses (Figure 1F).

3.2 | APAP-induced ALF produces severe coagulopathy

Consistent with previous studies [10,11,13], we observed a significant increase in plasma TAT complexes, indicative of coagulation activation, in APAP-challenged mice across all doses, and this was particularly notable at the highest dose tested (600 mg/kg) (Figure 2A). Similar findings were observed in a second independent study using a different TAT ELISA (Supplemental Figure S1). Plasma fibrinogen concentrations were unchanged in mice challenged with 300 or 450 mg/kg APAP (Figure 2B), but dramatically reduced in mice challenged with 600 mg/kg APAP (Figure 2B). Finally, plasma prothrombin concentrations were slightly but nonsignificantly reduced in mice challenged with 300 mg/kg APAP compared with saline-challenged mice, and significantly reduced in mice challenged with 600 mg/kg APAP compared with either saline- or 300 mg/kg APAP-treated mice (Figure 2C).

3.3 | Hepatic fibrin(ogen) deposition after the APAP challenge does not display an obvious dose–response

The deposition of cross-linked fibrin(ogen) is a hallmark of APAP-induced liver injury [20]. We anticipated that hepatic fibrin(ogen)

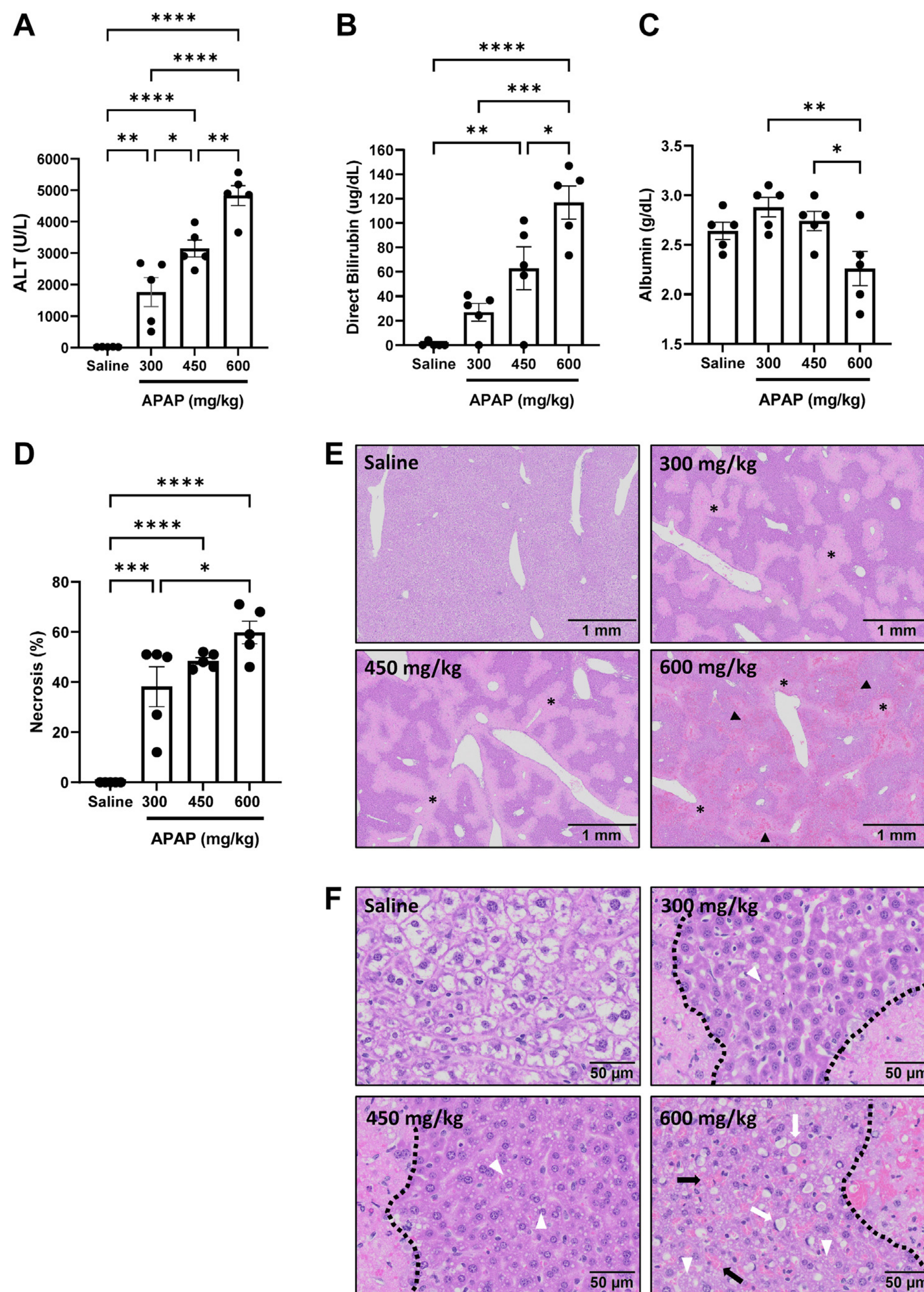

FIGURE 1 Assessment of APAP dose-dependent effects on liver injury and function. Fasted male wild-type mice were challenged with saline or APAP (300, 450, or 600 mg/kg i.p.), and samples were collected 24 hours later. Plasma (A) ALT, (B) direct bilirubin, and (C) albumin concentrations were measured. (D) Area of hepatocellular necrosis expressed as percentage of tissue area. (E) H&E staining of mouse livers. Necrotic area is indicated by black asterisk, sinusoidal congestion/hemorrhage by black triangle. (F) High magnification of liver H&E staining. Dashed line indicates outer area of necrotic area. Hepatocyte cytoplasmic vacuolation is indicated by white triangle, sinusoidal perturbation by black arrow, and hepatocyte ballooning by white arrow. Individual mice are plotted, bars represent mean ± standard error of mean. ALT, alanine aminotransferase; APAP, acetaminophen; HandE, hematoxylin and eosin; i.p. intraperitoneal. *p < .05, **p < .01, ***p < .001, ****p < .0001.

deposits would increase in a dose-dependent manner in mice challenged with APAP. As anticipated, hepatic deposits of insoluble cross-linked fibrin(ogen), demarked by cross-linked Fib(A)α and Fibγ, as well as Fib(B)β, were increased in the livers of APAP-challenged mice (Figure 3A–F) but displayed no obvious dose–response relationship. To determine if fibrin(ogen) also accumulated in other tissues, we used capillary western blotting to assess the concentrations of Fibβ in heart and kidney tissue after APAP overdose. Contrasting our expectations, we observed a dose-dependent decrease in insoluble fibrin(ogen) deposition in these tissues after the APAP challenge (Supplemental

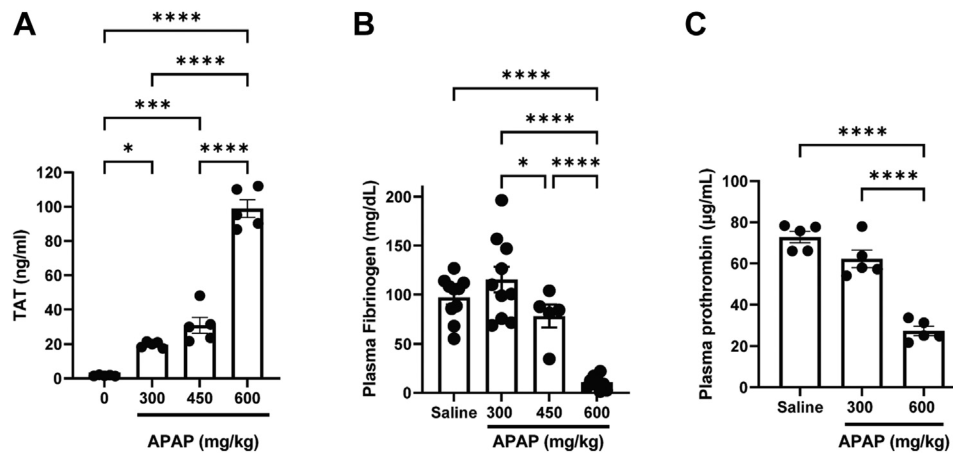

FIGURE 2 Assessment of APAP dose-dependent effects on plasma coagulation factors. Fasted male wild-type mice were challenged with saline or APAP (300, 450, or 600 mg/kg). Blood was collected 24 hours after challenge. (A) Plasma thrombin–anti-thrombin (TAT) complexes as a marker for coagulation activation. (B) Plasma fibrinogen antigen concentrations were measured by ELISA. (C) Plasma prothrombin concentrations were measured by ELISA. Values for individual mice are plotted, bars represent mean + SEM. APAP, acetaminophen. *$p < .05$, ***$p < .001$, ****$p < .0001$.

Figure S2). Notably, plasma fibrin(ogen) degradation products (FDPs, eg, D-dimer) increased dramatically in the plasma of mice challenged with 600 mg/kg APAP compared with mice treated with saline or lower doses of APAP (Figure 3G).

3.4 | Thrombin inhibition dramatically reduces consumption of coagulation factors in mice challenged with 600 mg/kg APAP

Our findings suggest that challenge with 600 mg/kg APAP elicits robust coagulation activation that promotes liver pathology. To test this hypothesis, we challenged mice with 600 mg/kg APAP and then treated mice with the direct thrombin inhibitor, DE, beginning 2 hours after the APAP challenge. Diluted thrombin time was used to estimate active DE concentration in plasma of saline-treated mice (Figure 4A, B). In APAP-challenged mice, treatment with DE significantly reduced plasma TAT concentrations (Figure 4C), preserved plasma fibrinogen concentrations (Figure 4D), and decreased plasma concentrations of FDPs (Figure 4E) compared with vehicle-treated mice. Previous studies have shown that treatment with anticoagulants, such as heparin and DE, before the 300 mg/kg APAP challenge reduces early APAP toxicity [10,13,14,27]. Consistent with these prior reports, we found that anticoagulation with DE also reduced hepatic necrosis (Figure 4G, black asterisks) and hemorrhage/congestion (black arrowheads) 24 hours after the 600 mg/kg APAP challenge (Figure 4F, G).

3.5 | Plasma from mice with APAP-induced ALF does not support thrombin-mediated clot formation *ex vivo*

Consistent with the observed reduction in plasma fibrinogen and prothrombin (Figure 2B, C), the PT was dramatically prolonged in plasma from mice challenged with 600 mg/kg APAP, whereas minimal change was evident in mice given 300 or 450 mg/kg APAP (Figure 5A). Restoring plasma fibrinogen to physiological concentrations (ie, 150 mg/dL) did not fully correct the PT in mice challenged with 600 mg/kg APAP (Figure 5B). Upon addition of TF, plasma from mice challenged with saline or 300 mg/kg APAP supported fibrin clot formation, monitored by increased turbidity in a microplate (Figure 5C, black and blue solid line, respectively). TF-induced coagulation did not increase turbidity in plasma from mice challenged with 600 mg/kg APAP (Figure 5C, orange solid line). Notably, plasma from mice challenged with 600 mg/kg APAP had a higher starting OD at 405 nm, potentially due to the buildup of products such as bilirubin (Supplemental Figure S3). To determine whether the loss of fibrin formation was caused by reduced fibrinogen (Figure 2B), we spiked fibrinogen into the plasma samples to normalize the starting concentration. However, *ex vivo* correction of fibrinogen to 200 mg/dL in plasma from mice challenged with 600 mg/kg did not enable fibrin formation (ie, turbidity) increase (Figure 5C, orange dotted line, Supplemental Figure S3). Although prothrombin concentrations were significantly reduced only in mice challenged with 600 mg/kg APAP, the APAP challenge suppressed plasma thrombin generation regardless of APAP dose (Table, Supplemental Figure S4, Supplemental Tables S1–S5). Surprisingly, even with normalization of plasma fibrinogen, addition of thrombin directly to plasma did not drive a fibrin clot formation in mice challenged with 600 mg/kg APAP (Figure 5D, Supplemental Figure S3). Notably, this defect could be overcome by increasing the concentration of thrombin added to plasma, suggesting the presence of an endogenous thrombin inhibitor (Figure 5E).

4 | DISCUSSION

Several studies have shown that standard APAP overdose in mice is associated with activation of coagulation [10,28]. However,

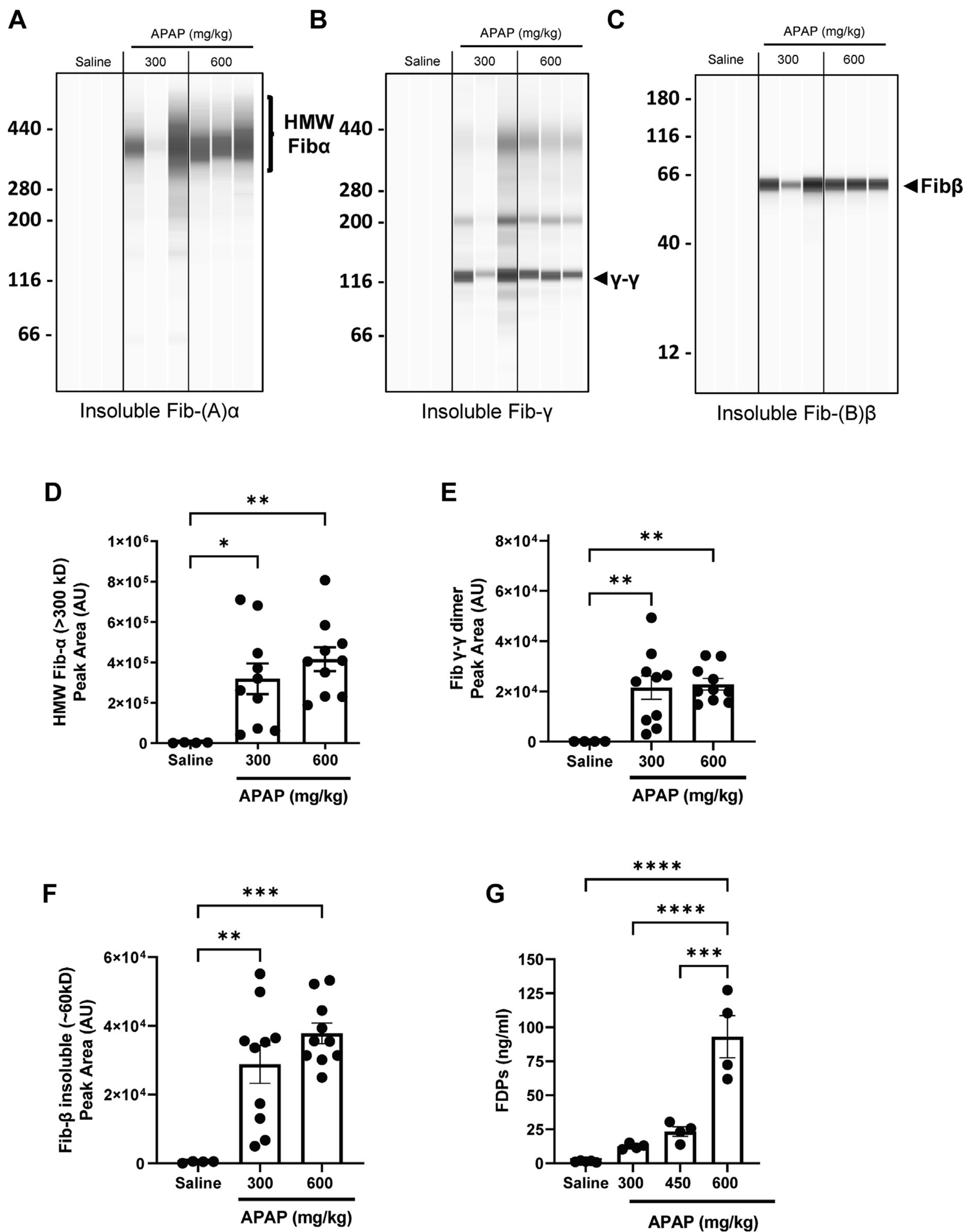

FIGURE 3 Assessment of dose-dependent effects of APAP on hepatic deposition of cross-linked fibrin(ogen). Fasted male mice were challenged with saline or APAP (300-600 mg/kg). Livers and blood samples were collected 24 hours later to assess hepatic fibrin(ogen) deposition and fibrin(ogen) degradation products, respectively. Fibrin(ogen) concentrations were measured in enriched insoluble liver extracts using automated capillary Western blotting (Wes by ProteinSimple). Representative digital capillary images show fibrin(ogen) detected by rabbit polyclonal antibodies selective for fibrin(ogen) Aα chain (A), γ chain (B), and Bβ chain (C). Quantification of peaks is shown in panels D–F. (G) Plasma concentrations of fibrin(ogen) degradation products (FDPs) measured using D-dimer ELISA. Individual mice are plotted, bars represent mean + SEM, n = 4-10 mice per group. APAP, acetaminophen. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$.

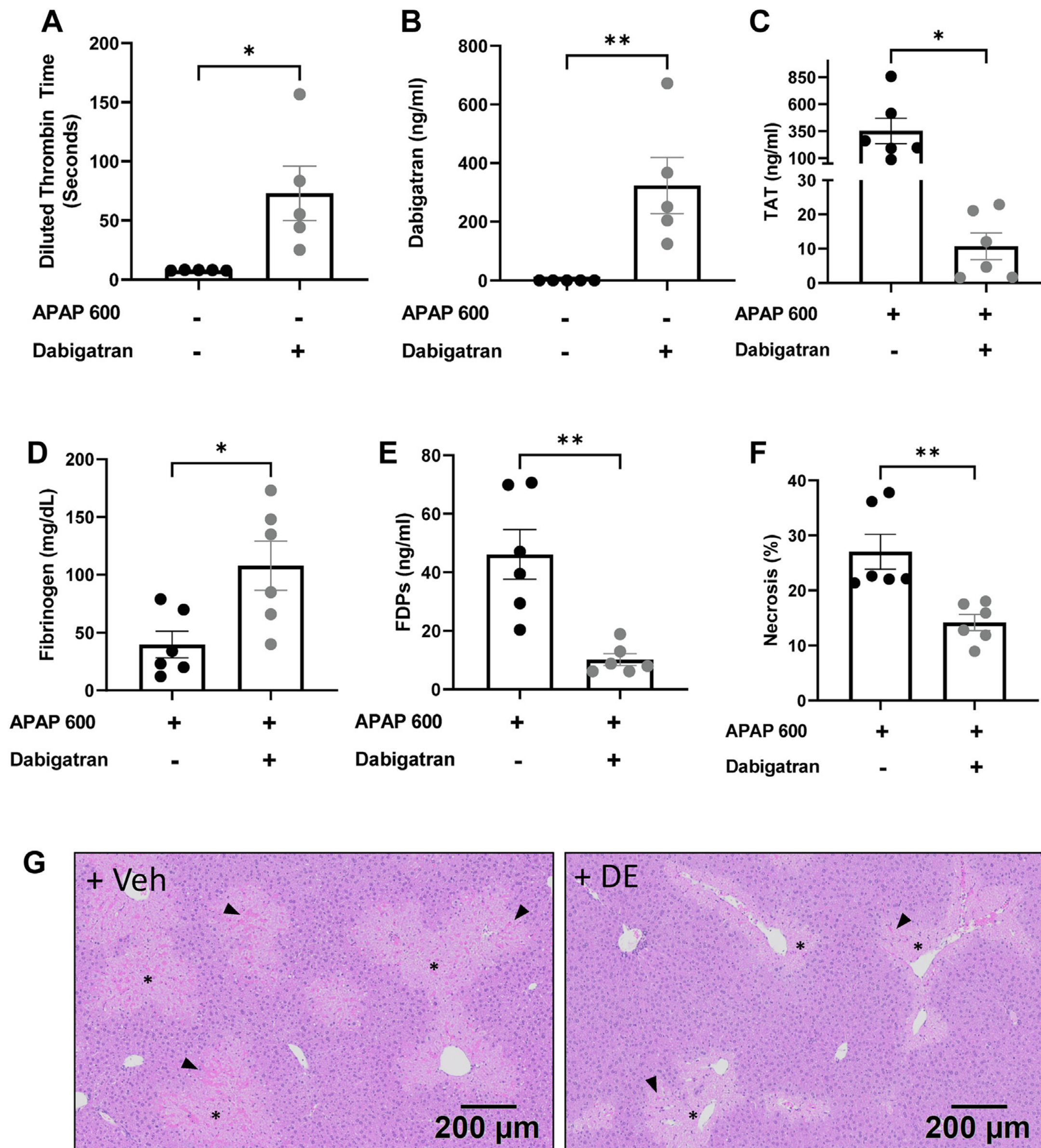

FIGURE 4 Impact of DE posttreatment on ALF-induced coagulopathy and liver injury. Fasted male wild-type mice were challenged with saline or 600 mg/kg APAP. To inhibit thrombin, mice were treated 2 and 12 hours after challenge with DE (10 mg/kg) or vehicle (2% dimethyl sulfoxide, 30% glycerinformal-cremophor). Samples were collected 24 hours after challenge to assess coagulation parameters and liver necrosis. To assure that this treatment paradigm resulted in meaningful DE plasma concentrations, diluted thrombin time was used to assess DE plasma concentrations in a separate group of mice 6 hours after vehicle (saline) treatment (A, B). Plasma thrombin–anti-thrombin (TAT) complexes (C), plasma fibrinogen (D), and plasma fibrin(ogen) degradation products (E) were measured 24 hours after challenge. Livers were collected 24 hours after APAP challenge to assess hepatocellular injury. (F) Area of hepatocellular necrosis, expressed as percentage. (G) Representative HandE staining of mouse livers of vehicle-treated (left) and DE-treated (right) mice. Necrotic area is indicated by black asterisk, sinusoidal congestion/hemorrhage by black triangle. Individual mice are plotted, bars represent mean+SEM. ALF, acute liver failure; APAP, acetaminophen; DE, dabigatran etexilate; HandE, hematoxylin and eosin. *$p < .05$, **$p < .01$.

experimental settings of APAP hepatotoxicity (ie, 300-450 mg/kg APAP) do not produce ALF-like pathology, and therefore, may not fully recapitulate the mechanisms driving changes in the hemostatic system in ALF patients. We identified apparent thresholds in the APAP dose–response wherein large doses of APAP that produce ALF-like pathology in mice [15] cause substantial changes in the hemostatic system. Changes observed in mice challenged with 600 mg/kg APAP included evidence of consumptive coagulopathy as evidenced by a substantially prolonged PT, near undetectable concentrations of plasma fibrinogen, and dramatic elevation in markers of coagulation activation (TAT) and fibrinolysis (fibrin degradation products). Notably, DE treatment attenuated changes in TAT and fibrin degradation products and produced hepatoprotective effects in a model otherwise associated with failed repair. These results highlight how

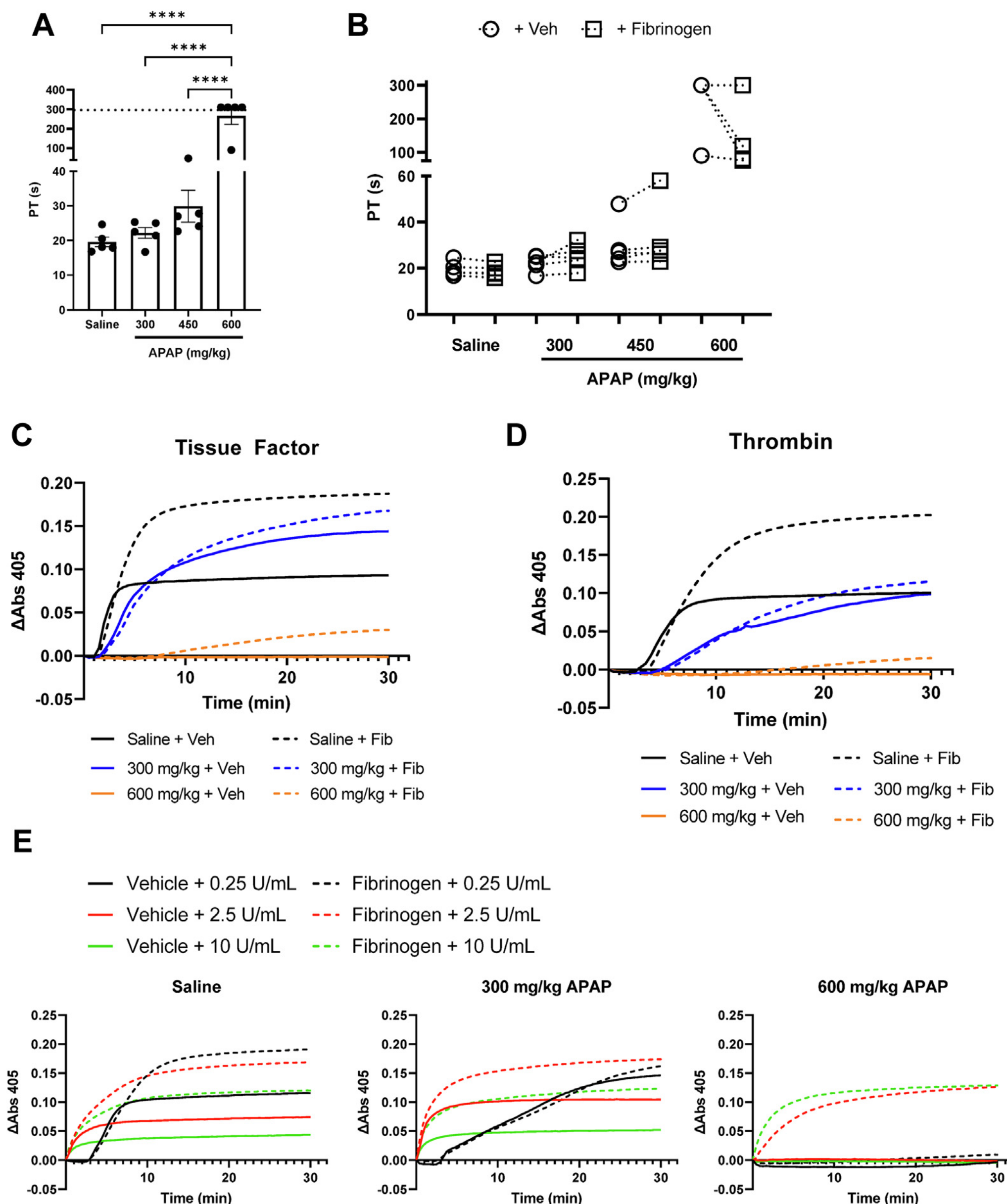

FIGURE 5 Assessment of dose-dependent effects of APAP on *ex vivo* plasma clot formation: Fasted male wild-type mice were challenged with saline or APAP (300, 450, or 600 mg/kg). Blood was collected 24 hours after challenge. (A) PT in plasma collected from saline or APAP-challenged mice. (B) Mouse fibrinogen or vehicle (Owren's Veronal buffer) was spiked into all plasma samples to achieve a final plasma fibrinogen concentration of 150 mg/dL and PT was subsequently measured. Note that plasma from 2 mice treated with 600 mg/kg APAP still failed to form a clot after addition of mouse fibrinogen (symbols overlap). Change in turbidity at 405 nm was monitored in plasma after clot formation was initiated with tissue factor (C), 0.25 U/mL thrombin (D), or various concentrations of thrombin (0.25-10 U/mL, E). Values for individual mice are plotted for A, B, and bars represent mean+SEM. Panels C,D show average turbidity curves obtained from 5 mice per group. Individual turbidity curves can be found in Supplemental Figure S3. APAP, acetaminophen; PT, prothrombin time. ****$p < .0001$.

APAP dose–response studies can be used to uncover the mechanisms of hemostatic changes associated with APAP hepatotoxicity and APAP-induced ALF.

Plasma TAT concentration was increased substantially, and plasma fibrinogen concentration reduced in mice challenged with 600 mg/kg APAP compared with lower APAP doses, suggesting marked coagulation activation. We were surprised that this dramatic decrease in plasma fibrinogen was not paired with a further increase in hepatic deposition of fibrin(ogen) in mice given 600 mg/kg APAP. One explanation for these observations is increased fibrin degradation, suggested by dramatic elevation of plasma FDPs in mice challenged with 600 mg/kg APAP. Additional studies monitoring biomarkers of plasmin production (eg, plasmin-antiplasmin complexes) will be required to solidify this conclusion. Alternatively, decreased hepatic function in mice challenged with 600 mg/kg APAP may suppress hepatic synthesis of fibrinogen, which could also lead to the observed

TABLE Plasma thrombin generation parameters in mice challenged with saline or 300, 450, or 600 mg/kg APAP.

Treatment	Lag time (min)	Time to peak (min)	Velocity (nM/min)	Peak (nM)	Endogenous thrombin potential (nM/min)
Saline	3.4 ± 0.4	5.6 ± 0.6	35.8 ± 9.1	76.0 ± 8.6	635.6 ± 32.1
300 mg/kg Acetaminophen	6.9 ± 3.4	10.6 ± 4.2	6.9 ± 7.8[a]	22.4 ± 20.2[a]	253.6 ± 188.6[a]
450 mg/kg Acetaminophen	22.6 ± 25.4	25.4 ± 24.0	2.0 ± 2.7[a]	8.5 ± 8.6[a]	107.3 ± 87.1[a]
600 mg/kg Acetaminophen	31.8 ± 28.2	33.9 ± 26.1	1.4 ± 1.4[a]	7.41 ± 7.4[a]	91.5 ± 92.3[a]

Coagulation was initiated in the presence of 1 pM tissue factor. The assay was performed in duplicate (N = 5 mice per group). Replicate values are shown for each parameter in Supplemental Tables S1–S5).

ETP, endogenous thrombin potential.

[a] $p < .05$ compared to saline-challenged mice.

reduction in plasma fibrinogen. Collectively, the results suggest that the reduction in plasma fibrinogen concentrations in mice with APAP-induced ALF reflects consumption and degradation of fibrin(ogen), as well as a possible decrease in fibrinogen synthesis by the injured liver.

Impaired TF- or thrombin-initiated clot formation in plasma from mice challenged with 600 mg/kg APAP was not fully corrected by addition of normal human fibrinogen. Notably, this defect could be overcome by increasing the concentration of thrombin added to the plasma in the presence of exogenous human fibrinogen. These studies suggest that an endogenous thrombin inhibitor impacts *ex vivo* coagulation of plasma from APAP-challenged mice. Although the precise mechanisms are not known, this observation aligns with prior studies describing a “heparin-like effect” in a porcine model of ALF, as well as in patients with ALF and advanced chronic liver disease [29–32]. Consequences of hepatic dysfunction, such as increased bilirubin concentration, may impact thrombin generation or fibrin polymerization. Moreover, marked endothelial damage in these disease settings is proposed to promote the release of endogenous heparin-like molecules, and syndecan-1 concentrations are increased in plasma of both mice and humans with acute APAP-induced liver injury [33]. Interestingly, endothelial injury and intrahepatic congestion/hemorrhage are among the key features distinguishing histopathology in mice challenged with 600 mg/kg APAP from lower APAP doses [17]. The apparent presence of an endogenous thrombin inhibitor adds to the complex list of changes affecting *ex vivo* coagulation in plasma from APAP-challenged mice, including marked reductions in plasma fibrinogen, which appears to exceed that observed in patients. It may also be useful in future studies to examine if changes in anticoagulant proteins, such as antithrombin or thrombomodulin, impact thrombin-generation parameters in APAP-challenged mice, as is the case in patients.

Changes in plasma-based coagulation assays evident in APAP-challenged mice are influenced by coagulation factor consumption. Prior studies have shown that coagulation activation after challenge with standard hepatotoxic doses of APAP is TF dependent [10,11], although the mechanisms driving exaggerated coagulation in mice challenged with 600 mg/kg are not known. Although TF in the liver (eg, hepatocellular TF) is a primary initiator of coagulation after APAP overdose, larger doses of APAP may drive excess coagulation by nonparenchymal cell TF activation. This may be another consequence of exaggerated endothelial cell damage evident in mice given the higher dose of APAP, perhaps connected to loss of function of anticoagulant pathways. Alternatively, an exaggerated inflammatory response could amplify coagulation. For example, a recent study described exaggerated production of interleukin-6 (IL-6) as a driver of pathologies observed in ALF in mice challenged with APAP, including disruption of cerebral blood flow [17]. It is also possible that amplification of coagulation by inflammatory cytokines like IL-6 occurs after APAP challenge. The precise mechanisms driving prothrombin consumption and increase in TAT require further investigation.

DE treatment after the 600 mg/kg APAP challenge significantly attenuated coagulation activity. Although there was no obvious reduction in serum ALT activity (not shown), DE treatment reduced the extent of liver necrosis. The results suggest that the coagulopathy observed in experimental APAP-induced ALF is pathologic and should fuel discussion on whether anticoagulation would benefit ALF patients, either by preventing liver damage or perhaps even the progression of extrahepatic pathologies. Histopathologic analysis revealed rare tubular casts (<1% tubules affected) and rare tubules lined by degenerate-to-necrotic epithelial cells in mice challenged with 600 mg/kg APAP and vehicle (1 mouse) or DE (1 mouse) (Supplemental Figure S5). These changes were deemed minor and likely without the consequences on renal function, but we cannot exclude progression of injury at time points not yet examined. Additional studies are required to weigh risks/benefits of pharmacologic anticoagulation, selection of an anticoagulant drug, and to identify the ideal timing of anticoagulation after APAP challenge. We selected to administer DE 2 hours after the APAP challenge. This avoids potential effects on APAP metabolism but does not directly address the question of ideal timing in a patient population. Additional studies uncovering the precise time course of coagulopathy after APAP challenge and defining the precise mechanisms linking coagulation activity to liver injury and repair will provide information on these key questions. Prior studies suggest that thrombin can exacerbate APAP hepatotoxicity by promoting hepatic platelet accumulation [13]. Substantial sinusoidal congestion was evident in the livers of mice treated with

600 mg/kg APAP, which appeared to be reduced in mice treated with DE. Due to either intrahepatic coagulation or excess plasmin production, severe sinusoidal congestion could impair hepatic blood flow, resulting in hypoxia and impaired liver repair after 600 mg/kg APAP [15]. Restoration of hepatic blood flow by anticoagulation may enable the clearance of debris by leukocytes and perhaps drive hepatocyte proliferation. Regardless of the exact mechanisms, this reduction in hepatic necrosis after DE treatment is an important finding as the 600 mg/kg APAP model is characteristically associated with a failure of the liver to repair [15].

In summary, we found that APAP-induced ALF in mice is associated with profound changes in the hemostatic system not evident in standard APAP hepatotoxicity, including a consumptive coagulopathy that appears to exacerbate hepatotoxicity and a heparin-like effect detectable using *ex vivo* clotting assays. These results suggest that experimental APAP dose–response studies could be used to uncover the mechanistic basis of hemostatic changes observed in ALF patients and could reveal novel pro-regenerative targets to stimulate liver repair.

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AUTHOR CONTRIBUTIONS

D.J.G., L.G.P., T.L., A.S.W., and J.P.L. contributed to the design of these studies. Experiments were performed and data were collected by D.J.G., E.G.B., C.Z.W., A.S., L.G.P., and K.J.W. Data were analyzed and interpreted by D.J.G., V.W., L.G.P., E.G.B., K.J.W., T.L., A.S.W., and J.P.L. The manuscript was drafted by D.J.G. All authors have reviewed and approved the final version of the manuscript for submission.

DECLARATION OF COMPETING INTERESTS

The authors have no relevant conflicts of interest to disclose.

DATA AVAILABILITY

For original data, please contact luyendyk@msu.edu.

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REFERENCES

[1] Lee WM. Acetaminophen-related acute liver failure in the United States. *Hepatol Res*. 2008;38:S3–8. https://doi.org/10.1111/j.1872-034X.2008.00419.x

[2] Larson AM, Polson J, Fontana RJ, Davern TJ, Lalani E, Hynan LS, Reisch JS, Schiødt FV, Ostapowicz G, Shakil AO, Lee WM, Group ALFS. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology*. 2005;42:1364–72. https://doi.org/10.1002/hep.20948

[3] Kerr R, Newsome P, Germain L, Thomson E, Dawson P, Stirling D, Ludlam CA. Effects of acute liver injury on blood coagulation. *J Thromb Haemost*. 2003;1:754–9. https://doi.org/10.1046/j.1538-7836.2003.00194.x

[4] Habib M, Roberts LN, Patel RK, Wendon J, Bernal W, Arya R. Evidence of rebalanced coagulation in acute liver injury and acute liver failure as measured by thrombin generation. *Liver Int*. 2014;34:672–8. https://doi.org/10.1111/liv.12369

[5] Stravitz RT, Fontana RJ, Meinzer C, Durkalski-Mauldin V, Hanje AJ, Olson J, Koch D, Hamid B, Schilsky ML, McGuire B, Ganger D, Liou I, Karvellas CJ, Rule JA, Lisman T, Clasen K, Reuben A, Cripps M, Lee WM, Group ALFS. Coagulopathy, bleeding events, and outcome according to rotational thromboelastometry in patients with acute liver injury/failure. *Hepatology*. 2021;74:937–49. https://doi.org/10.1002/hep.31767

[6] Stravitz RT, Lisman T, Luketic VA, Sterling RK, Puri P, Fuchs M, Ibrahim A, Lee WM, Sanyal AJ. Minimal effects of acute liver injury/acute liver failure on hemostasis as assessed by thromboelastography. *J Hepatol*. 2012;56:129–36. https://doi.org/10.1016/j.jhep.2011.04.020

[7] Driever EG, Stravitz RT, Zhang J, Adelmeijer J, Durkalski V, Lee WM, Lisman T. VWF/ADAMTS13 imbalance, but not global coagulation or fibrinolysis, is associated with outcome and bleeding in acute liver failure. *Hepatology*. 2021;73:1882–91. https://doi.org/10.1002/hep.31507

[8] Jaeschke H, Adelusi OB, Akakpo JY, Nguyen NT, Sanchez-Guerrero G, Umbaugh DS, Ding WX, Ramachandran A. Recommendations for the use of the acetaminophen hepatotoxicity model for mechanistic studies and how to avoid common pitfalls. *Acta Pharm Sin B*. 2021;11:3740–55. https://doi.org/10.1016/j.apsb.2021.09.023

[9] Groeneveld D, Cline-Fedewa H, Baker KS, Williams KJ, Roth RA, Mittermeier K, Lisman T, Palumbo JS, Luyendyk JP. Von Willebrand factor delays liver repair after acetaminophen-induced acute liver injury in mice. *J Hepatol*. 2020;72:146–55. https://doi.org/10.1016/j.jhep.2019.09.030

[10] Ganey PE, Luyendyk JP, Newport SW, Eagle TM, Maddox JF, Mackman N, Roth RA. Role of the coagulation system in acetaminophen-induced hepatotoxicity in mice. *Hepatology*. 2007;46:1177–86. https://doi.org/10.1002/hep.21779

[11] Sullivan BP, Kopec AK, Joshi N, Cline H, Brown JA, Bishop SC, Kassel KM, Rockwell C, Mackman N, Luyendyk JP. Hepatocyte tissue factor activates the coagulation cascade in mice. *Blood*. 2013;121:1868–74. https://doi.org/10.1182/blood-2012-09-455436

[12] Hugenholtz GC, Adelmeijer J, Meijers JC, Porte RJ, Stravitz RT, Lisman T. An unbalance between von Willebrand factor and ADAMTS13 in acute liver failure: implications for hemostasis and clinical outcome. *Hepatology*. 2013;58:752–61. https://doi.org/10.1002/hep.26372

[13] Miyakawa K, Joshi N, Sullivan BP, Albee R, Brandenberger C, Jaeschke H, McGill MR, Scott MA, Ganey PE, Luyendyk JP, Roth RA. Platelets and protease-activated receptor-4 contribute to acetaminophen-induced liver injury in mice. *Blood*. 2015;126:1835–43. https://doi.org/10.1182/blood-2014-09-598656

[14] Kopec AK, Joshi N, Cline-Fedewa H, Wojcicki AV, Ray JL, Sullivan BP, Froehlich JE, Johnson BF, Flick MJ, Luyendyk JP. Fibrin(ogen) drives repair after acetaminophen-induced liver injury via leukocyte αMβ2 integrin-dependent upregulation of Mmp12. *J Hepatol*. 2017;66:787–97. https://doi.org/10.1016/j.jhep.2016.12.004

[15] Bhushan B, Walesky C, Manley M, Gallagher T, Borude P, Edwards G, Monga SP, Apte U. Pro-regenerative signaling after acetaminophen-induced acute liver injury in mice identified using a

novel incremental dose model. *Am J Pathol*. 2014;184:3013–25. https://doi.org/10.1016/j.ajpath.2014.07.019
[16] Rama Rao KV, Verkman AS, Curtis KM, Norenberg MD. Aquaporin-4 deletion in mice reduces encephalopathy and brain edema in experimental acute liver failure. *Neurobiol Dis*. 2014;63:222–8. https://doi.org/10.1016/j.nbd.2013.11.018
[17] Roth K, Strickland J, Gonzalez-Pons R, Pant A, Yen T-C, Freeborn R, et al. Interleukin-6 drives key pathologic outcomes in experimental acetaminophen-induced liver failure. *bioRxiv*. 2021. https://doi.org/10.1101/2021.11.15.468664
[18] Sullivan BP, Kassel KM, Jone A, Flick MJ, Luyendyk JP. Fibrin(ogen)-independent role of plasminogen activators in acetaminophen-induced liver injury. *Am J Pathol*. 2012;180:2321–9. https://doi.org/10.1016/j.ajpath.2012.02.011
[19] Pant A, Kopec AK, Baker KS, Cline-Fedewa H, Lawrence DA, Luyendyk JP. Plasminogen activator inhibitor-1 reduces tissue-type plasminogen activator-dependent fibrinolysis and intrahepatic hemorrhage in experimental acetaminophen overdose. *Am J Pathol*. 2018;188:1204–12. https://doi.org/10.1016/j.ajpath.2018.01.010
[20] Poole LG, Kopec AK, Groeneveld DJ, Pant A, Baker KS, Cline-Fedewa HM, Flick MJ, Luyendyk JP. Factor XIII cross-links fibrin(-ogen) independent of fibrin polymerization in experimental acute liver injury. *Blood*. 2021;137:2520–31. https://doi.org/10.1182/blood.2020007415
[21] Gao S, Silasi-Mansat R, Behar AR, Lupu F, Griffin CT. Excessive plasmin compromises hepatic sinusoidal vascular integrity after acetaminophen overdose. *Hepatology*. 2018;68:1991–2003. https://doi.org/10.1002/hep.30070
[22] Shan Z, Li L, Atkins CL, Wang M, Wen Y, Jeong J, et al. Chitinase 3-like-1 contributes to acetaminophen-induced liver injury by promoting hepatic platelet recruitment. *eLife*. 2021;10:e68571. https://doi.org/10.7554/eLife.68571
[23] Joshi N, Kopec AK, Ray JL, Cline-Fedewa H, Nawabi A, Schmitt T, Nault R, Zacharewski TR, Rockwell CE, Flick MJ, Luyendyk JP. Fibrin deposition following bile duct injury limits fibrosis through an αMβ2-dependent mechanism. *Blood*. 2016;127:2751–62. https://doi.org/10.1182/blood-2015-09-670703
[24] Groeneveld D, Pereyra D, Veldhuis Z, Adelmeijer J, Ottens P, Kopec AK, Starlinger P, Lisman T, Luyendyk JP. Intrahepatic fibrin(ogen) deposition drives liver regeneration after partial hepatectomy in mice and humans. *Blood*. 2019;133:1245–56. https://doi.org/10.1182/blood-2018-08-869057
[25] Miszta A, Kopec AK, Pant A, Holle LA, Byrnes JR, Lawrence DA, Hansen KC, Flick MJ, Luyendyk JP, de Laat B, Wolberg AS. A high-fat diet delays plasmin generation in a thrombomodulin-dependent manner in mice. *Blood*. 2020;135:1704–17. https://doi.org/10.1182/blood.2019004267
[26] Tchaikovski SN, VAN Vlijmen BJ, Rosing J, Tans G. Development of a calibrated automated thrombography based thrombin generation test in mouse plasma. *J Thromb Haemost*. 2007;5:2079–86. https://doi.org/10.1111/j.1538-7836.2007.02719.x
[27] Kuwano A, Kohjima M, Suzuki H, Yamasaki A, Ohashi T, Imoto K, Kurokawa M, Morita Y, Kato M, Ogawa Y. Recombinant human soluble thrombomodulin ameliorates acetaminophen-induced liver toxicity in mice. *Exp Ther Med*. 2019;18:1323–30. https://doi.org/10.3892/etm.2019.7665
[28] Ward SE, O'Sullivan JM, Drakeford C, Aguila S, Jondle CN, Sharma J, Fallon PG, Brophy TM, Preston RJS, Smyth P, Sheils O, Chion A, O'Donnell JS. A novel role for the macrophage galactose-type lectin receptor in mediating von Willebrand factor clearance. *Blood*. 2018;131:911–6. https://doi.org/10.1182/blood-2017-06-787853
[29] Lee KCL, Baker L, Mallett S, Riddell A, Chowdary P, Alibhai H, Chang YM, Priestnall S, Stanzani G, Davies N, Mookerjee R, Jalan R, Agarwal B. Hypercoagulability progresses to hypocoagulability during evolution of acetaminophen-induced acute liver injury in pigs. *Sci Rep*. 2017;7:9347. https://doi.org/10.1038/s41598-017-09508-3
[30] Senzolo M, Agarwal S, Zappoli P, Vibhakorn S, Mallett S, Burroughs AK. Heparin-like effect contributes to the coagulopathy in patients with acute liver failure undergoing liver transplantation. *Liver Int*. 2009;29:754–9. https://doi.org/10.1111/j.1478-3231.2009.01977.x
[31] Shalaby S, Simioni P, Campello E, Spiezia L, Gavasso S, Bizzaro D, Cardin R, D'Amico F, Gringeri E, Cillo U, Barbiero G, Battistel M, Zanetto A, Ruzzarin A, Burra P, Senzolo M. Endothelial damage of the portal vein is associated with heparin-like effect in advanced stages of cirrhosis. *Thromb Haemost*. 2020;120:1173–81. https://doi.org/10.1055/s-0040-1713169
[32] Agarwal B, Wright G, Gatt A, Riddell A, Vemala V, Mallett S, Chowdary P, Davenport A, Jalan R, Burroughs A. Evaluation of coagulation abnormalities in acute liver failure. *J Hepatol*. 2012;57:780–6. https://doi.org/10.1016/j.jhep.2012.06.020
[33] Arnold K, Xu Y, Sparkenbaugh EM, Li M, Han X, Zhang X, et al. Design of anti-inflammatory heparan sulfate to protect against acetaminophen-induced acute liver failure. *Sci Transl Med*. 2020;12:eaav8075. https://doi.org/10.1126/scitranslmed.aav8075

SUPPLEMENTARY MATERIAL

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