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CRITICAL CARE

Anti-high-mobility group box-1 treatment strategies improve trauma-induced coagulopathy in a mouse model of trauma and shock

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Abstract

Background: Trauma-induced coagulopathy is associated with platelet dysfunction and contributes to early mortality after traumatic injury. Plasma concentrations of the damage molecule high-mobility group box-1 (HMGB-1) increase after trauma, which may contribute to platelet dysfunction. We hypothesised that inhibition of HMGB-1 with a monoclonal antibody (mAb) or with recombinant thrombomodulin (rTM) improves trauma-induced coagulopathy in a murine model of trauma and shock.

Methods: Male 129S2/SvPasOrlRJ mice were anaesthetised, mechanically ventilated, and randomised into five groups: (i) ventilation control (VENT), (ii) trauma/shock (TS), (iii) TS+anti-HMGB-1 mAb (TS+AB), (iv) TS+rTM (TS+TM), and (v) TS+anti-HMGB-1 mAb+rTM (TS+COMBI). Primary outcome was rotational thromboelastometry EXTEM. Secondary outcomes included tail bleeding time, platelet count, plasma HMGB-1 concentration, and platelet activation.

Results: Trauma and shock resulted in a hypocoagulable thromboelastometry profile, increased plasma HMGB-1, and increased platelet activation markers. TS+AB was associated with improved clot firmness after 5 min compared with TS (34 [33–37] *vs* 32 [29–34] mm; P=0.043). TS+COMBI was associated with decreased clot formation time (98 [92–125] *vs* 122 [111–148] s; P=0.018) and increased alpha angle (77 [72–78] *vs* 69 [64–71] degrees; P=0.003) compared with TS. TS+COMBI also reduced tail bleeding time compared with TS (P=0.007). The TS+TM and TS+COMBI groups had higher platelet counts compared with TS (P=0.044 and P=0.041, respectively).

Conclusions: Inhibition of HMGB-1 early after trauma in a mouse model improves clot formation and strength, preserves platelet count, and decreases bleeding time.

Keywords: coagulopathy; HMGB-1; inflammation; platelets; thromboelastometry; thrombomodulin; trauma

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Editor's key points

- Trauma-induced coagulopathy with platelet dysfunction contributes to early mortality after traumatic injury and might result from increased concentrations of the damage molecule high-mobility group box-1 (HMGB-1) after trauma.
- The authors used a mouse trauma model to determine whether inhibition of HMGB-1 with a monoclonal antibody or with recombinant thrombomodulin improves trauma-induced coagulopathy.
- Trauma and shock led to hypocoagulability, increased plasma HMGB-1 concentrations, and increased platelet activation that was mitigated by anti-HMGB-1 therapies.
- Early after trauma and shock, inhibition of HMGB-1 improves clot formation and clot strength and reduces bleeding time, which warrants further studies in longer-duration trauma models.

Bleeding after trauma is a leading cause of preventable mortality worldwide.[1–3] Up to 50% of severely injured patients develop trauma-induced coagulopathy (TIC), which is associated with increased early mortality.[4] Platelets are critical in the haemostatic response but can become dysfunctional within minutes after injury,[5,6] characterised by increased platelet activation but decreased ability to adhere and aggregate, thereby contributing to TIC and bleeding.[5,7–11] The mechanisms by which platelets become dysfunctional after trauma are largely unknown.

The damage-associated molecular pattern (DAMP) protein high-mobility group box-1 (HMGB-1) is released from damaged cells and platelets after trauma.[12–14] Circulating HMGB-1 binds to pattern recognition receptors, such as toll-like receptors and receptor for advanced glycation end products, on both platelets and immune cells, leading to platelet activation and (innate) immune activation.[13] Localised HMGB-1 release is necessary for normal platelet function, but disproportional systemic release of HMGB-1 is associated with development of TIC, increased platelet activation, and impaired platelet aggregation.[13–16]

HMGB-1 can be directly inhibited by monoclonal antibodies (mAb) but is also inactivated by thrombin–thrombomodulin complexes using recombinant thrombomodulin (rTM).[17–19] Pre-clinical studies have shown that inhibition of HMGB-1 after traumatic injury reduces the incidence of thromboembolic events and improves organ dysfunction,[13,20] but to date, the effect of HMGB-1 inhibition on early TIC has not been investigated. We studied the effects of anti-HMGB-1 mAb or rTM on TIC in a murine model of trauma and shock. We hypothesised that inhibition of HMGB-1 improves rotational thromboelastometry (ROTEM®; Werfen, Barcelona, Spain) coagulation parameters after traumatic injury.

Methods

Ethics and animals

Experiments were performed with approval of the Institutional Animal Care and Use Committee of the Amsterdam UMC, location AMC. Procedures were performed in accordance with the European Parliament directive (2010/63/EU) and the Dutch national law the Experiments on Animals Act. The study was pre-registered at preclinicaltrials.eu (PCTE0000247). All experiments were planned and performed according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.[21]

Male 129S2/SvPasOrlRJ mice (Janvier Labs, France) were housed in individually ventilated cages in the on-site animal housing facility for 7 days before experiments. The animals had access to food (Teklad global 16% protein; Envigo, Indianapolis, IN, USA) and water *ad libitum*, and they were exposed to regular 12 h day/night cycles. All mice were 8–10 weeks old during experiments.

Model of trauma-induced coagulopathy

Mice were anaesthetised in an induction chamber with sevoflurane 4.5 vol% (AbbVie, North Chicago, IL, USA) with FiO_2=0.6 and were injected with fentanyl 0.06 μg g^{-1} i.p. and then 0.03 μg g^{-1} i.p. after 30 min (hameln pharma, Germany). Tracheostomy was performed during mask ventilation (sevoflurane 4.5 vol%; FiO_2=0.5), after which mice were mechanically ventilated (VentElite; Harvard Apparatus, Holliston, MA, USA) with tidal volumes of 7.2 μl g^{-1}, ventilatory frequency of 190 bpm, inspiratory:expiratory ratio of 1:1.5, and FiO_2=0.4. A lung recruitment manoeuvre (inspiratory sigh of 20%) was performed every 30 min. Mice remained anaesthetised with sevoflurane 2.5 vol% throughout the experiment. Anaesthetic depth was confirmed every 10 min by the absence of pedal reflex and haemodynamic changes in response to a painful stimulus.

The right carotid artery was cannulated with a 2 French polyurethane catheter (Instech, Plymouth Meeting, PA, USA), which was connected to a pressure transducer and amplifier (Quad Bridge Amp; ADInstruments, Sydney, Australia). PowerLab version 4/30 and LabChart version 8 (ADInstruments) were used for data acquisition. The jugular vein was cannulated with a 1 French polyurethane catheter (Instech), after which mice received continuous fentanyl 0.12 μg g^{-1} h^{-1} i.v. (Pump 11 Pico Plus Elite; Harvard Apparatus) in Ringer's lactate 20 μl g^{-1} h^{-1} supplemented with glucose 15.3 mM and sodium bicarbonate 2 mM (B. Braun, Melsungen, Germany). Temperature was monitored with a rectal thermometer and kept at 37°C using a heated table and heat lamp.

After cannulation, mice were randomised using a block design to one of the following five groups (n=12 per group): ventilation+vehicle (VENT), trauma and shock+vehicle (TS), trauma and shock+anti-HMGB-1 mAb (TS+AB), trauma and shock+rTM (TS+TM), or trauma and shock+anti-HMGB-1 mAb+rTM (TS+COMBI). The operator was blinded to treatment allocation. In the trauma and shock groups, after randomisation, 200 μl blood was withdrawn through the carotid artery catheter, and body temperature was passively lowered to 35°C. Trauma consisted of bilateral lower-limb fractures using two haemostatic forceps. Midline laparotomy was performed to induce crush injury by clamping the small intestine distally of Treitz ligament five times for 2 s. Liver injury was created by clamping 1 cm of the left lobe for 2 s. The abdomen was closed with 6–0 silk suture (Ethicon, Raritan, New Jersey, USA). Consecutively, another 200 μl blood was withdrawn through the carotid artery catheter, followed by an infusion of Ringer's lactate 400 μl i.v. Successive blood draws were performed in 50–100 μl increments to induce shock with a target MAP of 20–25 mm Hg.

After induction of trauma and shock, mice received a bolus of purified anti-HMGB-1 mAb 1 μg g^{-1} i.v. (#B293347; low endotoxin, azide free; BioLegend, San Diego, CA, USA) or rTM 0.01 μg g^{-1} i.v. (#0909492; PeproTech, Rocky Hill, NJ, USA) or appropriate vehicles (phosphate-buffered saline [PBS] or PBS with bovine serum albumin 0.1%, respectively) in a total volume of 4 μl g^{-1}. At 30 min after drug infusion, blood was withdrawn through the carotid artery. Organ tissues were collected for analysis of wet/dry ratios, and lung was collected for pathological assessment (Supplementary methods). Pilots and dose–response studies preceded this experiment (Fig. 1a; Supplementary methods). Binding specificity of the antibody was confirmed *in vitro*, and the effect of immunoglobulin G on ROTEM was ruled out (Supplementary methods; Supplementary Fig. S2). An overview of the experimental set-up is shown in Fig. 2a.

Blood sampling

The first 50 μl blood was discarded because of saline contamination, after which 150 μl blood was collected in a heparin-coated syringe for arterial blood gas analysis (Siemens, Munich, Germany) and platelet count (Beckman Coulter, Brea, California, USA). The next 50 μl of blood was again discarded, and the remaining blood was collected in trisodium citrate 109 mM (1/9 v/v ratio). Part of the collected citrate-anticoagulated whole blood was used for ROTEM and flow cytometry. The remaining citrated blood was centrifuged twice (15 min, 2500 × *g* 20°C, acceleration 9, brake 3; centrifuge 5804R; rotor A-4-44; Eppendorf, Hamburg, Germany) and snap-frozen in liquid nitrogen before storage at −80°C until further analysis (Supplementary methods).

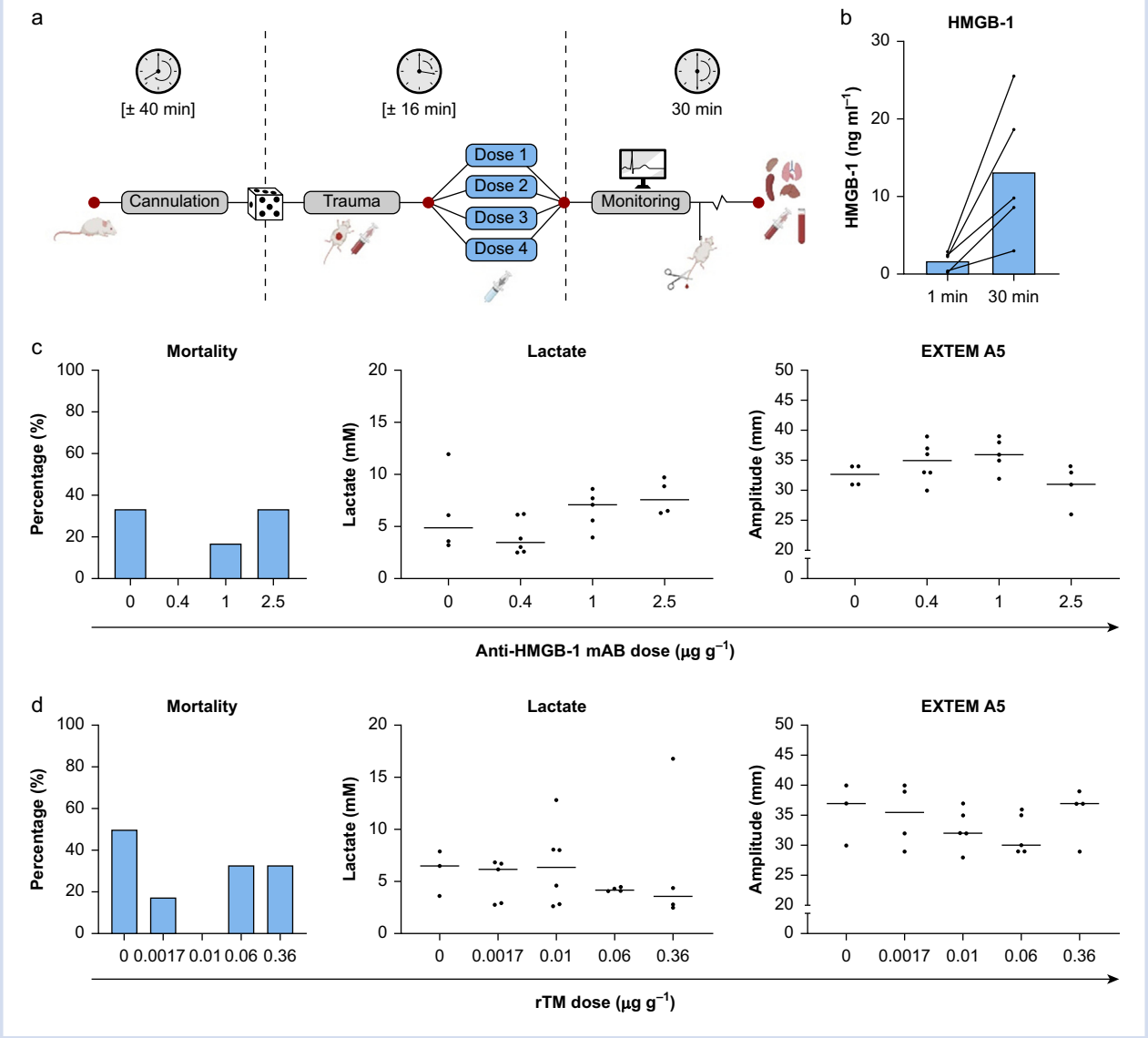

Fig 1. Dose–response studies. (a) Overview of dose–response studies. For anti-high-mobility group box-1 (HMGB-1) monoclonal antibody (mAb), four doses were tested. For recombinant thrombomodulin (rTM), five doses were tested. (b) Plasma HMGB-1 at 1 min after trauma *vs* 30 min after trauma. (c) HMGB-1 mAb dose–response. (d) rTM dose–response. Data points are shown with mean.

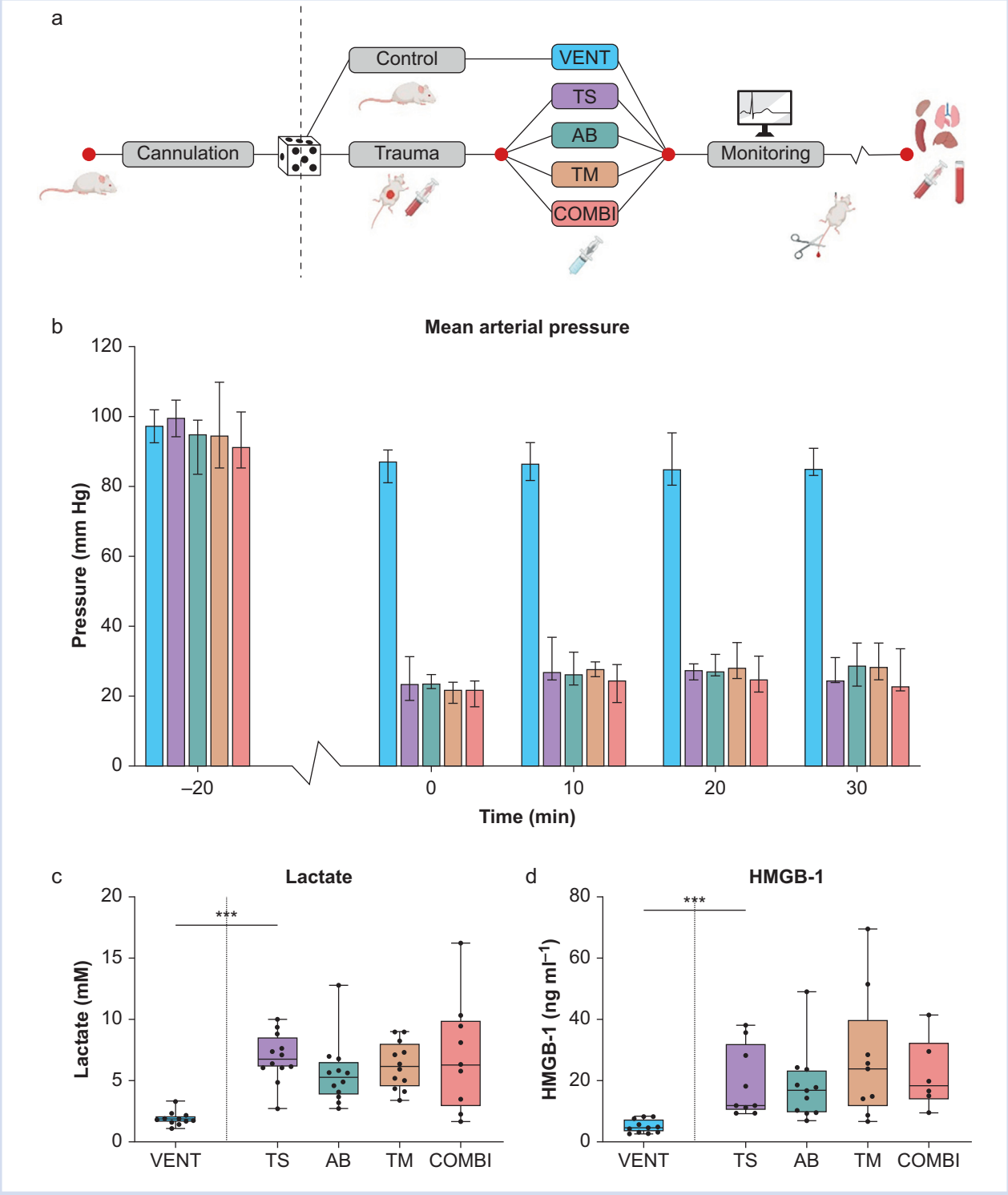

Fig 2. Shock and HMGB-1. (a) Murine model of trauma-induced coagulopathy. (b) MAP. Trauma and blood withdraw occurred between $t=-20$ min and $t=0$ min. Treatment or vehicle was administered at $t=0$ min, after which no more blood was withdrawn. The tail bleeding assay was performed at $t=10$ min. Mice were sacrificed at $t=30$ min. (c) Plasma lactate concentrations and (d) plasma HMGB-1 at the end of the experiment. AB, TS+anti-HMGB-1 mAb; COMBI, TS+anti-HMGB-1 mAb+rTM; HMGB-1, high-mobility group box-1; mAb, monoclonal antibody; rTM, recombinant thrombomodulin; TM, TS+rTM; TS, trauma and shock+vehicle; VENT, ventilation+vehicle. Data are presented as median (inter-quartile range) or as box plots (all data points are shown).***$P<0.001$ compared with TS.

Table 1 Shock characteristics and arterial blood gas analysis. HMGB-1, high-mobility group box-1; mAb, monoclonal antibody; rTM, recombinant thrombomodulin; TS, trauma and shock+vehicle; TS+AB, trauma and shock+anti-HMGB-1 mAb; TS+COMBI, trauma and shock+anti-HMGB-1 mAb+rTM; TS+TM, trauma and shock+rTM; VENT, ventilation+vehicle. Data are presented as median (inter-quartile range) or number (%). *P<0.05 compared with TS.

	VENT	TS	TS+AB	TS+TM	TS+COMBI
General					
Weight (g)	27.5 (25.8–28.7)	26.5 (25.3–29.2)	27.9 (25.1–29.3)	27.9 (26.4–28.6)	26.7 (25.9–27.4)
Blood withdrawn (μl)	0 (0–0)	630 (530–730)	650 (560–770)	630 (560–700)	630 (550–740)
Urine output (μl)	165 (150–238)	120 (78–188)	170 (105–230)	100 (35–223)	100 (90–118)
Mortality, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	2 (16.7)
Arterial blood gas analysis					
pH	7.34 (7.31–7.38)	7.33 (7.29–7.38)	7.30 (7.23–7.33)	7.32 (7.26–7.39)	7.20 (7.14–7.31)*
Pco_2 (kPa)	5.4 (5.0–6.0)*	3.5 (3.1–4.2)	3.9 (3.6–4.5)	3.6 (3.2–3.8)	3.6 (3.2–5.0)
Po_2 (kPa)	22.6 (20.8–24.8)*	27.3 (23.8–28.5)	24.0 (22.7–27.7)	26.4 (25.8–27.5)	24.6 (19.2–25.5)*
SaO_2 (%)	98.8 (98.5–99.0)	98.8 (98.5–98.9)	98.4 (98.2–98.7)	98.7 (98.2–99.0)	97.8 (97.0–98.4)*
HCO_3^- (mM)	21.5 (19.8–22.5)*	13.4 (12.7–15.8)	14.8 (13.8–16.4)	13.1 (12.3–15.8)	12.1 (8.6–18.0)
Base deficit (mM)	3.7 (2.7–4.3)*	11.1 (8.0–12.5)	11.1 (9.3–11.5)	10.8 (8.0–13.4)	14.6 (8.1–18.9)
Haemoglobin (mM)	8.7 (8.3–9.0)*	6.0 (5.4–6.5)	6.4 (6.0–6.6)	6.5 (6.4–7.2)*	6.4 (5.7–6.7)
K^+ (mM)	4.8 (4.5–5.0)*	6.4 (5.9–6.8)	6.5 (6.1–6.8)	6.1 (5.7–7.1)	6.2 (5.6–6.8)
Ca^{2+} (mM)	1.01 (0.99–1.17)	1.06 (0.96–1.13)	1.09 (1.05–1.12)	1.02 (0.96–1.11)	0.99 (0.95–1.07)
Glucose (mM)	5.9 (5.4–6.1)*	10.0 (7.7–14.1)	9.9 (7.9–12.7)	9.5 (7.6–11.1)	10.2 (8.2–12.5)

Rotational thromboelastometry

ROTEM EXTEM was performed using ROTEM minicups (Werfen, Barcelona, Spain) according to the manufacturer's guidelines. The EXTEM assay measures the tissue factor pathway by addition of EXTEM reagent 7 μl (containing tissue factor and phospholipids) and STARTEM 7 μl (containing calcium ions) to 105 μl citrated whole blood. Clotting time (CT) is the time until the clot reaches 2 mm in amplitude. Clot formation time (CFT) is the time between the 2 mm amplitude and the 20 mm amplitude. The alpha (α) angle represents the angle between the baseline and the tangent through the 2 mm point. The amplitude at 5 min (A5) represents the clot firmness 5 min after CT. Maximum clot firmness (MCF) shows the maximum amplitude. Lysis index at 60 min (LI60) is the percentage of maintained clot compared with MCF 60 min after CT.

Tail bleeding assay

At 20 min before the end of the experiment, the distal end of the tail (at 3 mm diameter) was cut off using a double guillotine cigar cutter.[22] The tail was submerged in pre-warmed (37°C) NaCl 0.9% (50 ml), and bleeding time was recorded.

Platelet activation and aggregation

For platelet activation measurement, citrated whole blood was diluted 20-fold in modified Tyrode's buffer and incubated for 15 min on a polypropylene 96-well plate (Corning, Corning, NY, USA) with allophycocyanin (APC) anti-CD41 antibody (BioLegend) with either fluorescein isothiocyanate (FITC) anti-CD62P (P-selectin; BD Biosciences, Franklin Lakes, NJ, USA) or phycoerythrin (PE) anti-glycoprotein IIb/IIIa (JON/A; Emfret Analytics, Eibelstadt, Germany). Adenosine diphosphate (ADP) 20 μM (Merck, Rahway, NJ, USA) was added for certain conditions. For measurement of platelet–leucocyte aggregates, citrated whole blood was incubated for 15 min with an antibody mixture containing APC anti-CD41 antibody with FITC anti-CD45 (BioLegend) and PE anti-Ly6G antibody (Thermo Fisher, Waltham, MA, USA).

Platelet aggregation was measured using flow cytometry, as described.[23] In short, whole blood was incubated for 15 min with either APC anti-CD41 antibody or PE anti-CD61 antibody (BD Biosciences). Excess antibody was removed by two washing steps. The two stained and washed conditions were mixed and incubated at 37°C for 15 min with ADP 20 μM or protease-activated receptor 4 activating peptide 100 μM (Cayman Chemical, Ann Arbor, MI, USA) while aggregating at 600 rpm. In both activation and aggregation assays, we focused on ADP activation and ADP/PAR4-induced aggregation based on the platelet dysfunction seen after traumatic injury.[5] All flow cytometry analyses were performed using the FACSCanto™ system (BD Biosciences). Antibody panels can be found in Supplementary Table S1.

Plasma HMGB-1 and fibrinogen

Plasma HMGB-1 and fibrinogen concentrations were measured using enzyme-linked immunosorbent assays according to the manufacturer's instructions (Elabscience, Houston, TX, USA).

Sample size calculation

Based on prior experiments, we determined group sizes of nine mice were needed to detect a clinically relevant difference in ROTEM parameters between the treatment groups and TS (V=6.5 and SD=3.6) with an α=0.05 and a power of 80%. To account for maximally 25% mortality in our model, 12 mice were used per group.

Statistical analysis

Data were analysed using SPSS version 25.0 (IBM, Armonk, NY, USA). Graphs were made using GraphPad Prism version 9.0 (San Diego, CA, USA). All data were assumed non-parametric and are presented as median with inter-quartile range, unless specifically stated otherwise. Outliers were identified using the ROUT test (Q=1%) and not included in statistical analysis. Non-parametric data were analysed with Kruskal–Wallis test, and

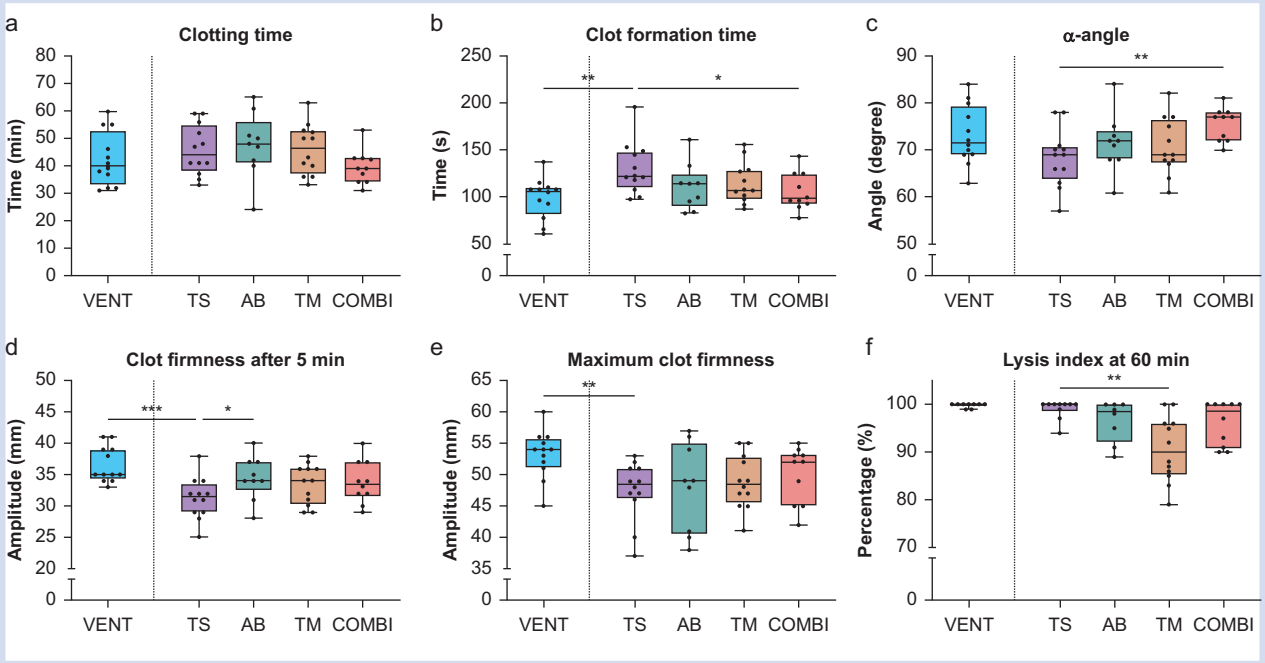

Fig 3. Rotational thromboelastometry. (a) Rotational thromboelastometry (ROTEM) EXTEM clotting time, (b) EXTEM clot formation time, (c) EXTEM alpha angle, (d) EXTEM clot firmness after 5 min, (e) EXTEM maximum clot firmness, and (f) EXTEM lysis index at 60 min. AB, TS+anti-HMGB-1 mAb; COMBI, TS+anti-HMGB-1 mAb+rTM; HMGB-1, high-mobility group box-1; mAb, monoclonal antibody; rTM, recombinant thrombomodulin; TM, TS+rTM; TS, trauma and shock+vehicle; VENT, ventilation+vehicle. Data are shown as box plots (all data points are shown). *P<0.05, **P<0.01, ***P<0.001 compared with TS.

predefined *post hoc* comparisons (VENT *vs* TS and TS *vs* treatment groups) were analysed with Dunn's test. Binominal data were analysed with Fisher's exact test. Tail bleeding time was analysed with the log-rank test. Because of the exploratory nature, no corrections were made for multiple testing. A *P*-value of <0.05 was considered to be statistically significant.

Results

Dose–response studies

Plasma HMGB-1 concentration increased 30 min after trauma and shock (9.8 [5.8–22.1] *vs* 2.3 [0.2–2.7] ng ml^{-1}; *P*=0.008; Fig. 1b). The dose–response results of anti-HMGB-1 mAb and rTM on mortality, lactate, and EXTEM A5 are shown in Fig. 1c and d. Blood gas analysis, additional ROTEM parameters, and organ wet/dry ratios are shown in Supplementary Tables S2 and S3. For anti-HMGB-1 mAb, both the vehicle and the highest dose showed higher mortality and decreased clotting amplitudes. Given this observation, a dose of 1 μg g^{-1} was chosen for subsequent experiments. rTM activates protein C in a dose-dependent manner.[24] A dose of 0.01 μg g^{-1} was chosen because it did not trigger fibrinolysis and was associated with the lowest mortality compared with other dosages (Supplementary Table S3).

Outcomes with anti-HMGB-1

Shock, HMGB-1, and mortality

The volume of blood withdrawn to reach a predefined MAP after trauma was similar between groups (Table 1). The model resulted in severe shock, as reflected by reduced arterial pressure and increased lactate concentration (6.8 [6.1–8.6] *vs* 1.8 [1.6–2.2] mM; *P*<0.001) in TS compared with VENT (Fig. 2b and c). HMGB-1 increased in TS compared with VENT (*P*<0.001) but did not differ between treatment groups and TS (Fig. 2d). Results from blood gas analysis are shown in Table 1. Both pH and Po_2 were decreased in TS+COMBI compared with TS (*P*=0.037 and *P*=0.04, respectively). Mortality only occurred in the TS+COMBI group (16.7%) because of deterioration of shock.

Trauma-induced coagulopathy

TS resulted in a pronounced coagulopathy compared with VENT, consisting of increased CFT, decreased clot firmness, and lower platelet counts (Figs. 3a–f and 4a–c).

TS+AB improved ROTEM A5 compared with TS (34 [33–37] *vs* 32 [29–34] mm; *P*=0.043) (Fig. 3d). TS+TM did not improve ROTEM parameters but showed increased fibrinolysis, as measured by LI60 (90 [85–96]%) compared with TS (100 [95–100]%; *P*=0.02; Fig. 3f). In the TS+COMBI group, there was reduced CFT (98 [92–125] *vs* 122 [111–148] s; *P*=0.018) and increased α angle (77 [72–78] *vs* 69 [64–71] degrees; *P*=0.003) compared with TS (Fig. 3a–c). In contrast to TS+TM, TS+COMBI was not associated with increased fibrinolysis compared with TS.

TS+TM and TS+COMBI preserved platelet counts compared with TS (Fig. 4a). Fibrinogen did not differ between groups (Fig. 4b). The TS+COMBI group had shorter tail bleeding time compared with TS (*P*=0.007) (Fig. 4c).

Platelet activation and aggregation

TS induced an increase in platelet–leucocyte aggregates compared with VENT, mostly consisting of platelet–neutrophil

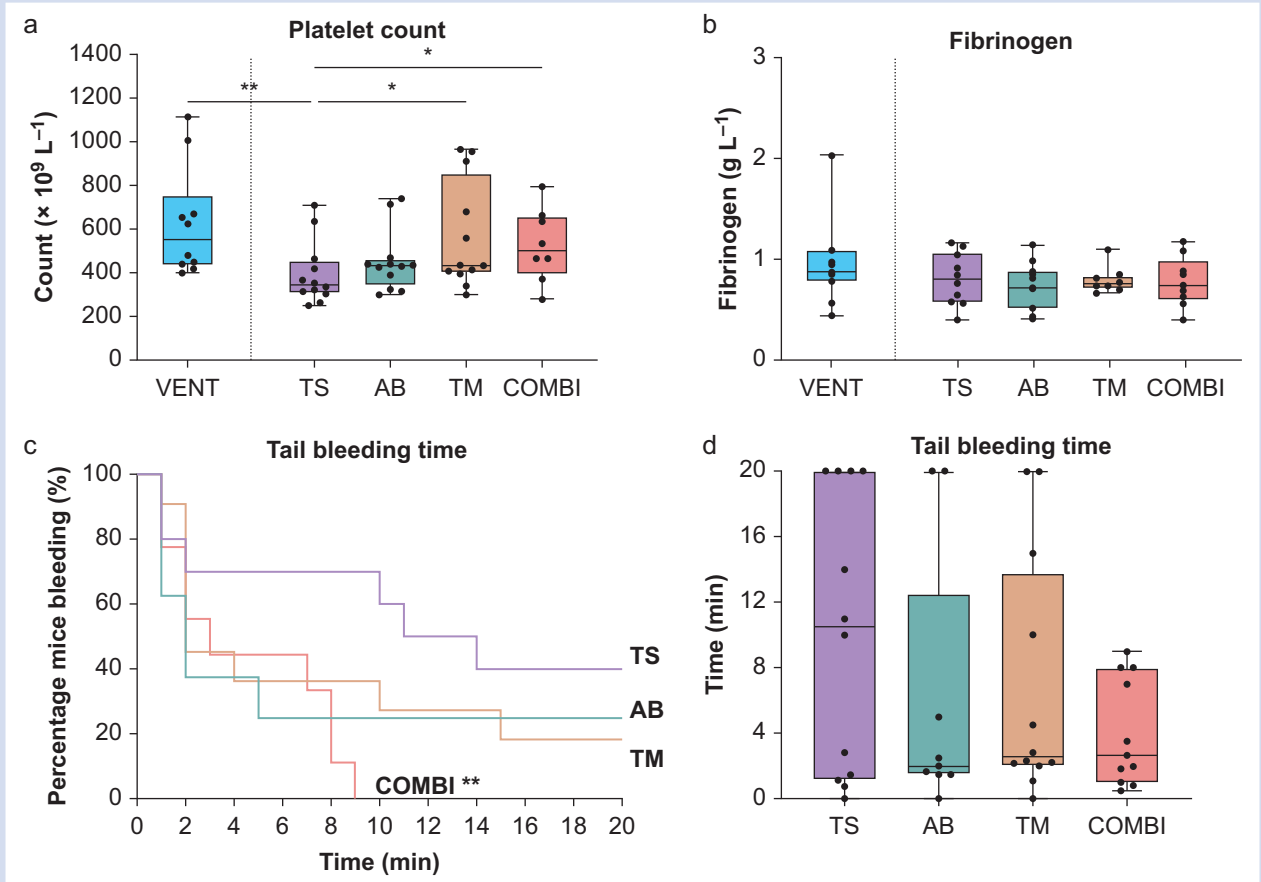

Fig 4. Conventional assays of coagulation (a) platelet count, (b) plasma fibrinogen, and (c–d) Tail bleeding time. AB, TS+anti-HMGB-1 mAb; COMBI, TS+anti-HMGB-1 mAb+rTM; HMGB-1, high-mobility group box-1; mAb, monoclonal antibody; rTM, recombinant thrombomodulin; TM, TS+rTM; TS, trauma and shock+vehicle; VENT, ventilation+vehicle. Data are shown as box plots (all data points are shown). *P<0.05, **P<0.01 compared with TS.

complexes (Fig. 5a), whereas P-selectin expression on single platelets was not increased (Fig. 5b). TS also increased platelet surface glycoprotein (GP) IIb/IIIa expression compared with VENT. Stimulation with ADP increased GP IIb/IIIa expression, but no differences were observed between groups (Fig. 5b and c). Anti-HMGB-1 treatment did not affect platelet activation. Platelet aggregation was neither impaired in TS compared with VENT, nor did it show improvement in the treatment groups compared with TS (Fig. 5d).

Inflammation, organ oedema, and lung injury

Plasma interleukin (IL)-6 concentration did not increase in TS compared with VENT, and plasma IL-1β concentration remained undetectable (<91.6 pg ml^{-1}; Supplementary Table S4). TS+COMBI was associated with lower IL-6 compared with TS. Both soluble intercellular adhesion molecule-1 and soluble P-selectin were lower in TS compared with VENT, but they did not differ between TS and treatment groups (Supplementary Table S4). Organ oedema determined by wet/dry ratios was not different between groups (Supplementary Table S4). Lung injury was absent in all groups, with only two animals scoring mild signs of haemorrhage (TS) and interstitial inflammation (TS+TM) (Supplementary Fig. S3).

Discussion

This trauma and shock model resulted in TIC with prolonged CFT and decreased clot firmness determined by ROTEM, and decreased platelet counts and increased platelet activation. Our study further showed that plasma concentration of HMGB-1 increased within the first 30 min after injury, which is in line with clinical data showing elevated HMGB-1 within hours of traumatic injury.[12,25,26] Inhibition of HMGB-1 improved ROTEM characteristics, preserved platelet counts, and decreased tail bleeding time with some distinct differences between treatments. Treatment with anti-HMGB-1 mAb was associated with an improvement in ROTEM A5. However, increasing the dose decreased clot firmness and increased mortality, demonstrating the necessity of some localised HMGB-1 release for normal platelet function.[27]

rTM preserved platelet counts but did not significantly improve ROTEM parameters. Reduced platelet counts in trauma are associated with worse outcomes, although platelet counts generally do not drop severely.[5,28] Additionally, normal

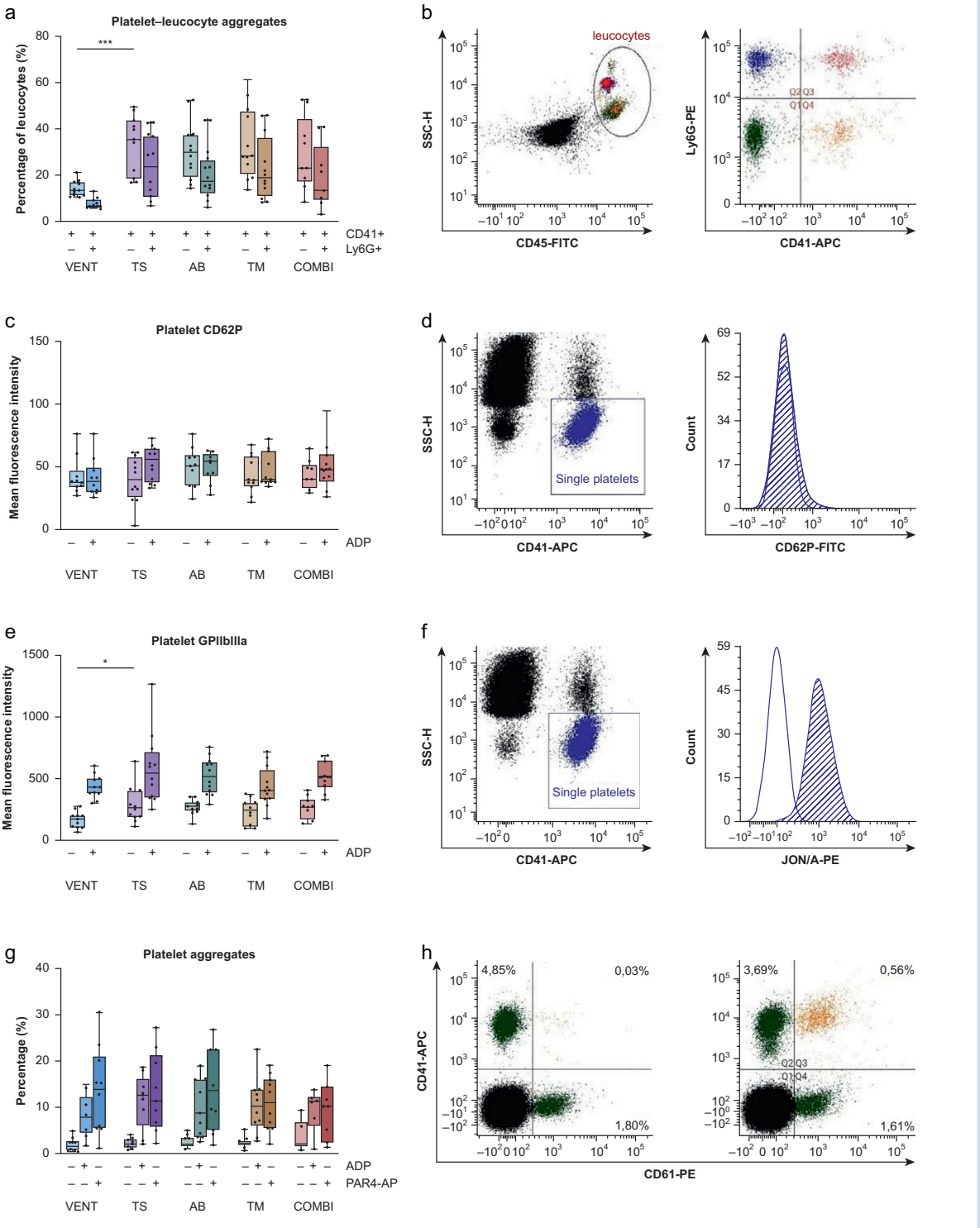

Fig 5. Platelet flow cytometry. (a) Platelet–leucocyte aggregates and platelet–neutrophil aggregates, displayed as a percentage of total leucocytes (CD45+). (b) Leucocytes were identified based on CD45+ and side scatter height (SSC-H) characteristics (Q1–4). Platelet–leucocyte aggregates were identified based on additional CD41+ (Q3+Q4); platelet–neutrophils were identified based on additional CD41+ and Ly6G+ (Q3). (c) Expression of CD62P (P-selectin) on platelet surface without stimulation and with adenosine diphosphate (ADP) 20 μM. (d) Single platelets were identified based on CD41/SSC-H characteristics. (e) Expression of GP IIb/IIIa on platelet surface without stimulation and with ADP 20 μM. (f) Single platelets were identified based on CD41/SSC-H characteristics. (g) Platelet aggregates without stimulation, with ADP 20 μM and with protease-activated receptor 4 activating peptide (PAR4-AP) 100 μM. (h) Two populations were stained with an antibody against CD41 or CD61. Platelet aggregates are calculated by dividing the double-stained events (Q3) by the total stained events (Q2–4). AB, TS+anti-HMGB-1 mAb; COMBI, TS+anti-HMGB-1 mAb+rTM; HMGB-1, high-mobility group box-1; mAb, monoclonal antibody; rTM, recombinant thrombomodulin; TM, TS+rTM; TS, trauma and shock+vehicle; VENT, ventilation+vehicle. Data are presented as box plots (all data points are shown). *P<0.05, ***P<0.001 compared with TS.

platelet counts in mice are higher compared with humans. It therefore remains to be determined whether rTM also preserves platelet counts in human trauma patients. In contrast to our dose–response study, we observed an increase in late fibrinolysis with the current dose of rTM, which might aggravate bleeding after trauma. However, this increased fibrinolysis did not lead to slower clot formation, lower amplitudes, or prolonged tail bleeding time.

Besides inactivation of HMGB-1 by thrombin–thrombomodulin complexes, thrombomodulin exerts other functions that could explain the observed platelet-preserving effects.[29] We propose that rTM protects platelets from damage either by limiting the HMGB-1–platelet interaction, by inhibiting other DAMPs (e.g. histones and extracellular DNA), or by reducing thrombin-induced cell damage. Initially, it might be counter-intuitive to administer rTM early because of its effects on protein C activation, which has been proposed as a key mechanism of TIC.[30] *In vitro* data suggest that low-dose rTM stimulates thrombin-activatable fibrinolysis inhibitor (TAFI) activation, which could be beneficial in aiding the reduction of hyperfibrinolysis in early TIC.[24,31] An optimised dose of rTM for traumatic shock should be based on TAFI *vs* protein C activation in future studies. Of note, we used human TM in this model, which might activate mouse protein C less efficiently than murine TM.[32] However, we performed dose–response studies to determine an adequate dose for our mouse model.

Combining anti-HMGB-1 mAb with rTM appeared the most efficacious with regard to coagulation, as this therapy not only improved CFT and α angle, but it also resulted in preserved platelet counts and reduced tail bleeding time. Combining treatments, in contrast to rTM alone, did not result in increased fibrinolysis. Mechanistically, it can be hypothesised that anti-HMGB-1 neutralises HMGB-1, whereas rTM exerts more of its other functions, such as neutralisation of histones and limiting thrombin-induced cell damage, thereby limiting TIC. Of note, as mortality only occurred in the COMBI group, survival bias might explain (part of) the benefits on TIC parameters. Also, a lower pH and Po_2 compared with the TS group was observed in this group. However, we did not find any signs of lung injury or thrombus formation. Therefore, this study does not offer a definitive mechanistic explanation, was potentially too short, and not designed to assess adverse events of anti-HMGB-1 treatments.

Despite the observed benefits of anti-HMGB-1 treatments, they did not affect HMGB-1 concentration after 30 min. An explanation could be that anti-HMGB-1 treatments block the interaction between platelets and HMGB-1.[33] Alternatively, a single dose of anti-HMGB-1 mAb or rTM might not reduce circulating HMGB-1 after 30 min as HMGB-1 is released continuously during shock.

Although platelet GP IIb/IIIa expression and platelet–leucocyte aggregates were increased after trauma, anti-HMGB-1 treatment did not affect these activation markers. It is, however, unknown how the activation of circulating platelets relates to platelet function (i.e. aggregation).[6] Using an experimental assay for platelet aggregation, we were not able to detect impaired aggregation after trauma, and therefore, no improvements in platelet aggregation with anti-HMGB-1 treatments were observed. Because of low sample volume, we were also unable to evaluate platelet adhesion, which is known to be impaired after trauma.[9] As fibrinogen concentrations were comparable, the improvements in ROTEM characteristics with anti-HMGB-1 are likely because of improved platelet function.

One of the strengths of our model is the short duration, in which it mostly resembles pre-hospital conditions before in-hospital resuscitation. Besides the hypocoagulable state, we showed increased platelet–leucocyte aggregates, low expression of P-selectin on the surface of single platelets, and low circulating soluble P-selectin after trauma and shock. Previous literature in humans has shown increased P-selectin on platelets after traumatic injury and a higher concentration of soluble P-selectin in trauma patients compared with healthy volunteers.[5,34] This discrepancy might be time-related, as these coagulation assays are usually performed at later time points. We hypothesise that early on after trauma, cell-bound P-selectin is used in the formation of platelet–leucocyte aggregates. Over time, P-selectin-positive platelets detach from leucocytes, and P-selectin may be degraded.[35]

Given the short duration of our model, we did not evaluate microthrombus formation or the effects of anti-HMGB-1 treatments on late organ dysfunction. TIC characteristics change over time towards a more hypercoagulable state that could require time-dependent dosing of anti-HMGB-1 mAB and rTM.[36,37] Efficacy and safety in this regard remain to be determined in longer-duration trauma models.

This study has several limitations. First, experiments were performed in male mice, so differences in outcomes between sexes will require further study. Our model consisted of homogeneous tissue injury and controlled blood withdrawal to induce shock, which differs from the diverse tissue injuries and uncontrolled active bleeding in humans. Furthermore, anaesthesia dampens sympathetic activation, which is likely more pronounced in human trauma patients. We chose to solely evaluate the effect of anti-HMGB-1 treatments, which differs from clinical practice where resuscitation is regularly started within 30 min. The effects of anti-HMGB-1 strategies shown here could therefore be different in combination with standard resuscitation strategies.

In conclusion, HMGB-1 increases early after trauma and shock. Inhibition of HMGB-1 improves clot formation and clot strength within this model. Future studies should focus on the effects of anti-HMGB-1 treatment in longer-duration trauma models to address safety and interactions with current resuscitation strategies.

Authors' contributions

Study design: PHS, NPJ, DJBK.
Data collection: PHS, MAWM, JJTHR.
Statistical analysis: PHS, DJBK.
Data interpretation: PHS, JCMM, RN, JJTHR, NPJ, DJBK.
Drafting and revision of paper: PHS, DJBK.
Revision and final approval of paper: all authors.

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Declaration of interest

All authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2023.01.026.

References

1. GBD 2017 Causes of Death Collaborators. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet* 2018; **392**: 1736–88
2. Oyeniyi BT, Fox EE, Scerbo M, Tomasek JS, Wade CE, Holcomb JB. Trends in 1029 trauma deaths at a level 1 trauma center: impact of a bleeding control bundle of care. *Injury* 2017; **48**: 5–12
3. Koh EY, Oyeniyi BT, Fox EE, et al. Trends in potentially preventable trauma deaths between 2005–2006 and 2012–2013. *Am J Surg* 2019; **218**: 501–6
4. Brohi K, Singh J, Heron M, Coats T. Acute traumatic coagulopathy. *J Trauma* 2003; **54**: 1127–30
5. Kutcher ME, Redick BJ, McCreery RC, et al. Characterization of platelet dysfunction after trauma. *J Trauma Acute Care Surg* 2012; **73**: 13–9
6. Sloos PH, Vulliamy P, van 't Veer C, et al. Platelet dysfunction after trauma: from mechanisms to targeted treatment. *Transfusion* 2022; **62**: S281–300
7. St John AE, Newton JC, Martin EJ, et al. Platelets retain inducible alpha granule secretion by P-selectin expression but exhibit mechanical dysfunction during trauma-induced coagulopathy. *J Thromb Haemost* 2019; **17**: 771–81
8. Vulliamy P, Gillespie S, Armstrong PC, Allan HE, Warner TD, Brohi K. Histone H4 induces platelet ballooning and microparticle release during trauma hemorrhage. *Proc Natl Acad Sci U S A* 2019; **116**: 17444–9
9. Vulliamy P, Montague SJ, Gillespie S, et al. Loss of GPVI and GPIbα contributes to trauma-induced platelet dysfunction in severely injured patients. *Blood Adv* 2020; **4**: 2623–30
10. Ramsey MT, Fabian TC, Shahan CP, et al. A prospective study of platelet function in trauma patients. *J Trauma Acute Care Surg* 2016; **80**: 726–32
11. Wohlauer MV, Moore EE, Thomas S, et al. Early platelet dysfunction: an unrecognized role in the acute coagulopathy of trauma. *J Am Coll Surg* 2012; **214**: 739–46
12. Peltz ED, Moore EE, Eckels PC, et al. HMGB1 is markedly elevated within 6 hours of mechanical trauma in humans. *Shock* 2009; **32**: 17–22
13. Vogel S, Bodenstein R, Chen Q, et al. Platelet-derived HMGB1 is a critical mediator of thrombosis. *J Clin Invest* 2015; **125**: 4638–54
14. Cohen MJ, Brohi K, Calfee CS, et al. Early release of high mobility group box nuclear protein 1 after severe trauma in humans: role of injury severity and tissue hypoperfusion. *Crit Care* 2009; **13**: R174
15. Vogel S, Rath D, Borst O, et al. Platelet-derived high-mobility group box 1 promotes recruitment and suppresses apoptosis of monocytes. *Biochem Biophys Res Commun* 2016; **478**: 143–8
16. Ito T, Kawahara K, Nakamura T, et al. High-mobility group box 1 protein promotes development of microvascular thrombosis in rats. *J Thromb Haemost* 2007; **5**: 109–16
17. Abeyama K, Stern DM, Ito Y, et al. The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel antiinflammatory mechanism. *J Clin Invest* 2005; **115**: 1267–74
18. Ito T, Kawahara K, Okamoto K, et al. Proteolytic cleavage of high mobility group box 1 protein by thrombin-thrombomodulin complexes. *Arterioscler Thromb Vasc Biol* 2008; **28**: 1825–30
19. Zhou H, Wang Y, Wang W, et al. Generation of monoclonal antibodies against highly conserved antigens. *PLoS One* 2009; **4**, e6087
20. Yang Z, Simovic MO, Edsall PR, et al. HMGB1 inhibition to ameliorate organ failure and increase survival in trauma. *Biomolecules* 2022; **12**: 101
21. Percie du Sert N, Hurst V, Ahluwalia A, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLoS Biol* 2020; **18**, e3000410
22. Liu Y, Jennings NL, Dart AM, Du XJ. Standardizing a simpler, more sensitive and accurate tail bleeding assay in mice. *World J Exp Med* 2012; **2**: 30–6
23. Bonnard T, Law LS, Tennant Z, Hagemeyer CE. Development and validation of a high throughput whole blood thrombolysis plate assay. *Sci Rep* 2017; **7**: 2346
24. Mosnier LO, Meijers JC, Bouma BN. Regulation of fibrinolysis in plasma by TAFI and protein C is dependent on the concentration of thrombomodulin. *Thromb Haemost* 2001; **85**: 5–11
25. Giannoudis PV, Mallina R, Harwood P, Perry S, Sante ED, Pape HC. Pattern of release and relationship between HMGB-1 and IL-6 following blunt trauma. *Injury* 2010; **41**: 1323–7
26. Polito F, Cicciu M, Aguennouz M, et al. Prognostic value of HMGB1 and oxidative stress markers in multiple trauma patients: a single-centre prospective study. *Int J Immunopathol Pharmacol* 2016; **29**: 504–9
27. Yang X, Wang H, Zhang M, Liu J, Lv B, Chen F. HMGB1: a novel protein that induced platelets active and aggregation via Toll-like receptor-4, NF-κB and cGMP dependent mechanisms. *Diagn Pathol* 2015; **10**: 134
28. Hamada SR, Garrigue D, Nougue H, et al. Impact of platelet transfusion on outcomes in trauma patients. *Crit Care* 2022; **26**: 49
29. Watanabe-Kusunoki K, Nakazawa D, Ishizu A, Atsumi T. Thrombomodulin as a physiological modulator of intravascular injury. *Front Immunol* 2020; **11**, 575890
30. Brohi K, Cohen MJ, Ganter MT, et al. Acute coagulopathy of trauma: hypoperfusion induces systemic anticoagulation and hyperfibrinolysis. *J Trauma* 2008; **64**: 1211–7. discussion 7
31. van Moorsel MVA, Poolen GC, Koekman CA, et al. VhH anti-thrombomodulin clone 1 inhibits TAFI activation and enhances fibrinolysis in human whole blood under flow. *J Thromb Haemost* 2022; **20**: 1213–22
32. Raife TJ, Dwyre DM, Stevens JW, et al. Human thrombomodulin knock-in mice reveal differential effects of human thrombomodulin on thrombosis and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2011; **31**: 2509–17
33. Ahrens I, Chen YC, Topcic D, et al. HMGB1 binds to activated platelets via the receptor for advanced glycation end products and is present in platelet rich human coronary artery thrombi. *Thromb Haemost* 2015; **114**: 994–1003

34. Tang N, Yin S, Sun Z, Pan Y. Time course of soluble P-selectin and von Willebrand factor levels in trauma patients: a prospective observational study. *Scand J Trauma Resusc Emerg Med* 2013; **21**: 70
35. Panicker SR, Mehta-D'souza P, Zhang N, Klopocki AG, Shao B, McEver RP. Circulating soluble P-selectin must dimerize to promote inflammation and coagulation in mice. *Blood* 2017; **130**: 181–91
36. Dyer MR, Chen Q, Haldeman S, et al. Deep vein thrombosis in mice is regulated by platelet HMGB1 through release of neutrophil-extracellular traps and DNA. *Sci Rep* 2018; **8**: 2068
37. Sloos PH, Maas MAW, Hollmann MW, Juffermans NP, Kleinveld DJB. The effect of shock duration on trauma-induced coagulopathy in a murine model. *Intensive Care Med Exp* 2022; **10**: 1

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