Published in final edited form as:
Crit Care Med. 2013 November ; 41(11): e301–e308. doi:10.1097/CCM.0b013e31828a4520.

Effects of fibrinogen concentrate after shock/resuscitation – A comparison between in vivo microvascular clot formation and thromboelastometry

Judith Martini, MD[1], **Pedro Cabrales, PhD**[2], **Dietmar Fries, MD**[3], **Marcos Intaglietta, PhD**[2], and **Amy G. Tsai, PhD**[2]

[1]Department of Anesthesia and Intensive Care Medicine, Medical University Innsbruck, Anichstr. 35, 6020 Innsbruck. Austria

[2]Department of Bioengineering, University of California, San Diego (UCSD) 9500 Gilman Drive, La Jolla, CA, USA

[3]Clinical Department of General and Surgical Intensive Care Medicine, Medical University Innsbruck, Anichstr. 35, 6020 Innsbruck, Austria

Abstract

Objective—Dilutional coagulopathy after resuscitation with crystalloids/colloids clinically often appears as diffuse microvascular bleeding. Administration of fibrinogen reduces bleeding and increases maximum clot firmness (MCF), measured by thromboelastometry. Study objective was to implement a model where microvascular bleeding can be directly assessed by visualizing clot formation in microvessels, and correlations can be made to thromboelastometry.

Design—Randomized animal study.

Setting—University research laboratory.

Subjects—Male Syrian Golden hamsters.

Interventions—Microvessels of Syrian Golden hamsters fitted with a dorsal window chamber were studied using videomicroscopy. After 50% hemorrhage followed by 1 hr of hypovolemia resuscitation with 35% of blood volume using a high molecular weight (MW) HES solution (Hextend®, Hospira, MW 670 kD) occurred. Animals were then treated with 250 mg/kg fibrinogen iv (Laboratoire français du Fractionnement et des Biotechnologies (LFB), Paris, France) or an equal volume of saline before venular vessel wall injuries were made by directed laser irradiation and the ability of microthrombus formation was assessed.

Measurements and main results—Thromboelastometric measurements of MCF were performed at the beginning and at the end of the experiment. Resuscitation with HES and sham treatment significantly decreased FIBTEM MCF from 32 ± 9 at baseline vs. 13 ± 5 mm after sham

Address for reprints: Judith Martini, MD, Department of Anesthesia and Intensive Care Medicine, Medical University Innsbruck, Anichstr. 35, 6020 Innsbruck, Austria, Judith.Martini@uki.at.

The here presented work has been conducted at the Microhemodynamics Laboratory, Dept. of Bioengineering at UCSD.

Reprints will be ordered.

DF receives consulting and lecture honoraria from LFB. All other authors have no competing financial interests.

treatment ($p < 0.001$). Infusion of fibrinogen concentrate significantly increased MCF, restoring baseline levels (baseline 32 ± 9 mm; after fibrinogen administration 29 ± 2 mm).

In vivo microthrombus formation in laser injured vessels significantly increased in fibrinogen treated animals compared with sham (77% vs. 18%).

Conclusions—Fibrinogen treatment leads to increased clot firmness in dilutional coagulopathy as measured with thromboelastometry. At the microvascular level this increased clot strength, corresponds to an increased incidence of thrombus formation in vessels injured by focused laser irradiation.

Keywords

Dilutional coagulopathy; fibrinogen polymerization; laser injury; thrombus formation; hemorrhagic shock; colloid resuscitation

INTRODUCTION

The clinical scenario of dilutional coagulopathy accounts for a pronounced increase in early mortality of polytraumatized, severely bleeding patients (1–4). Resuscitation fluids, especially colloidal plasma expanders, which are used to restore normovolemia and thus guarantee perfusion pressure in vital organs, dilute clotting factors thereby leading to a diffuse, microvascular bleeding tendency (5–8). Animal (9–12) and clinical investigations (13, 14) showed that the presence of colloids decrease clot firmness due to direct interaction with fibrin polymerization by inducing changes to the ratio between fibrin fiber mass and fibrin length (6). Under these circumstances, plasma fibrinogen concentrations reach critical levels at an early stage and before any other coagulation factor. Interestingly, nearly 70% of lethal exsanguinations in trauma patients occur after hospital admission (15, 16) which could partly be attributed to coagulation disturbances.

Numerous studies have shown the effect of fibrinogen concentrate in reversal of dilutional coagulopathy after shock/resuscitation (12–14, 17–19). Nevertheless, in clinical practice it is not possible to monitor and measure microvascular bleeding in real time. In the perioperative and trauma-management setting, thromboelastometry has been implemented as a “point of care” diagnostic tool, which acquires information about clot quality in terms of clot firmness and clot stability over time and has been shown to rapidly identify patients requiring massive transfusion (20). However, thrombus formation is a dynamic process, including the interaction between fibrinogen and platelets, and between erythrocytes, leukocytes, microparticles (21) and being influenced by local blood flow and blood endothelial interactions (22). Thromboelastometry assesses clot formation and functionality; however, it does not provide information on the interaction amongst these factors *in vivo*.

The current study was performed in the hamster window chamber, an intravital microscopy model used for direct investigation of microvascular blood flow, capillary perfusion and *in vivo* visualization of thrombus formation. The assessment of these parameters in concert allows for a more physiological approach to understanding the mechanisms of clot formation under normal and pathological conditions such as shock/resuscitation. This study analyzed for the first time the complex interplay between blood flow conditions and

hemostasis during dilutional coagulopathy and directly assessed the effect of fibrinogen concentrate on microvascular thrombus formation *in vivo*.

MATERIALS AND METHODS

Animal preparation

Investigations were performed in 50 to 65 g male golden Syrian hamsters (Charles River Laboratories, Boston, MA) fitted with a dorsal window. Animal handling and care were provided following the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the study was approved by the local Animal Subjects Committee. The hamster window chamber model is widely used for microvascular studies in the unanesthetized state and allows for noninvasive measurements over extended periods of time of an intact vasculature without exposure to ambient air or need for perfusates (23, 24).

The complete surgical technique for the preparation has been previously described in detail (25, 26). Briefly, hamsters were prepared for chamber implantation with a 50 mg/kg ip injection of pentobarbital sodium anesthesia. After hair removal, sutures were used to lift the dorsal skin away from the animal, and one frame of the chamber was positioned on the animal's back. A chamber consisted of two identical titanium frames with a 15 mm circular window. With the aid of backlighting and a stereomicroscope, one side of the skinfold was removed following the outline of the window until only a thin layer of retractor muscle and the intact subcutaneous skin of the opposing side remained. Saline and then a cover glass were placed on the exposed skin held in place by the other part of the chamber. The intact skin of the other side was exposed to the ambient environment. The animal was allowed at least 2 days for recovery; its chamber was then assessed under the microscope for any signs of edema, bleeding, or unusual neovascularization. Barring these complications the animal was anesthetized again and arterial and venous catheters (polyethylene-50) were implanted in the carotid artery and jugular vein, respectively. Catheters were prefilled with saline, tunneled under the skin, exteriorized at the dorsal side of the neck and attached to the chamber frame with tape.

Inclusion criteria—Animals were evaluated for inclusion into the study 4 days after the initial surgery. Animals were suitable for the experiments if: *1*) systemic parameters were within normal range, namely, heart rate (HR) > 320 beats/min, mean arterial blood pressure (MAP) > 80 mm Hg, systemic hematocrit (HCT) > 45%, arterial PO_2 > 50 mmHg; and, *2*) microscopic examination of the tissue observed under x40 magnification did not show signs of edema or bleeding.

Intravital microscopy setup—The unanesthetized animal was placed into a restraining tube for the period of the experiment. The tube containing the conscious animal was fixed to the stage of a transillumination intravital microscope (BX51 W1, Olympus, New Hyde Park, NY). Animals had 30 min to adjust to the tube environment prior to measuring baseline parameters. The tissue image was projected onto a CCD camera (4815-2000, COHU, San Diego, CA) connected to a timer and viewed on a closed circuit monitor. Microvascular diameter and red blood cell (RBC) velocity were measured online in arterioles and venules

using a 40x water immersion objective (LUMPFL-WOR, numerical aperture 0.7, Olympus, Central Valley, PA). Blood flow rates were calculated from evaluated diameters and velocities as previously reported (27). The same vessel sites were followed throughout the entire experiment so that direct comparison could be made to baseline levels.

Experimental protocol—Figure 1 shows the experimental timeline and measurement time points. Baseline measurements (systemic parameters, blood gases and microhemodynamic parameters) were followed by an acute hemorrhage (withdrawal of 50% of estimated total blood volume (BV), calculated as 7% of the body weight) via the carotid artery catheter within five minutes. Hypovolemia was maintained for one hour before a single volume infusion (35% BV) of hydroxyethyl starch (Hextend®, Hospira Inc., Lake Forest, IL, mean MW 670 kDa; degree of substitution 0.75) occurred within ten minutes via the jugular vein catheter. Animals did not receive additional fluids during the experiment. Previous investigations performed in the same model have shown that fluid resuscitation with 25% of total blood volume is adequate since the mechanism of autotransfusion restores about half of the shed volume during shock (28). In this study however, 35% of total blood volume resuscitation was chosen to increase the volume effect and thereby produce severe dilutional coagulopathy.

In order to maximize the negative effect of the colloidal solution on the coagulation system and to allow the full clinical picture of dilutional coagulopathy to develop, animals remained in this resuscitated state for one hour. Then animals were randomized into two groups: 1.) Treatment, 250 mg/kg fibrinogen iv (FGTW, Laboratoire français du Fractionnement et des Biotechnologies (LFB) Paris, France), or, 2.) Sham, treatment with iv infusion of an equal volume of normal saline. The dose of 250 mg/kg of fibrinogen concentrate was chosen due to the results of preliminary experiments (unpublished) indicating a normalization of ROTEM® parameters after shock/resuscitation in this model. The fibrinogen concentrate and the control volume (normal saline) were infused at a rate of 0.2 ml/min.

Fifteen minutes after fibrinogen or saline administration, laser injuries were induced by targeting the beam of a dye laser (425 nm, 25 mW, Power Technology, Alexander, AR) on venular vessel walls. The laser beam was introduced via an optical port to an upright microscope (BX51 W1, Olympus, New Hyde Park, NY). A micropoint laser system (Photonics Instruments, St. Charles, IL) and a 60x objective (LUMPFL-WIR, numerical aperture 0.8, Olympus, Central Valley, PA) was used to collimate the beam. The beam was focused on the targeted vessel wall at 1/25 of the power for 5 sec. Before, during and after laser exposure, the vessel image was visualized using a video microscope system. After laser exposure the vessel was observed for one minute and the presence or absence of a thrombus was recorded. The event of thrombus formation was noted as “successful” when a flow disturbance was observed. Investigators were not blinded to the randomization status of the animal, which is a weakness of the study.

Cabrales *et al.* showed (28) that high blood flow rates prevented injury to the arteriolar vessel wall when laser exposure times were bellow 20 – 30 sec; however, in venules where the flow rates are much lower exposure of only 2 – 5 sec induced full thrombus development. Since the awake animal might move during such long exposure times, thereby

shifting the laser beam out of focus, only venules were used in this study. Thrombus formation was readily identified on the site of injury using transillumination without the use of platelet staining. Results were quantified by calculating the ratio of number of vessels with microthrombi divided by the total number of vessels exposed to irradiation.

Experimental groups

1. Microvascular analysis and thromboelastometry—In each animal, microvessels were chosen for study based on their type (arteriole or venule), vessel diameter, blood flow and visual clarity. Following these inclusion criteria, 8 – 11 venules and 4 – 6 arterioles were selected from each animal (*N*) (Treatment: $N = 5$, number of animals and Sham: $N = 5$). Microvascular parameters and thrombus formation were studied in total 98 venules (Treatment: *n*, number of vessels = 48 and Sham: $n = 50$). Additionally, microcirculation measurements were also performed in 45 arterioles (Treatment: $n = 22$ and Sham: $n = 23$). Blood was sampled from all animals in both groups for thromboelastometric evaluation.

2. Plasma fibrinogen concentration and platelet count—Changes of the plasma fibrinogen concentration and platelet count due to the experimental protocol was studied in another group of animals because of blood volume sampling limitations in this size of animal. For this additional analysis, animals ($N = 14$) underwent exactly the same surgical procedures, were randomized for 1.) Treatment: $N = 7$ or 2.) Sham: $N = 7$. Blood for platelet and fibrinogen concentration measurements was withdrawn at the end of the experimental protocol.

Measured parameters

a) Thromboelastometry—Coagulation measurements were performed by using a thromboelastometer (ROTEM® Gamma, Tem Innovations GmbH; Munich, Germany) to assess functional coagulation parameters: The device measures the elasticity of the developing and resolving clot over time which is used to assess all phases of clot formation, stabilization and degradation (29). Maximum clot firmness (MCF) is the most important parameter for this study since it is directly dependent on fibrinogen levels. ROTEM® assays were run at 37 °C immediately after drawing arterial blood in citrated 1ml syringes (0.04 ml of citrate/1 ml of whole blood). Due to the small total blood volume of the hamsters (between 3.5 – 4.5ml) and the relatively large sample size needed for ROTEM® analysis (300 μl/test), only two ROTEM® measurements could be performed in each animal. Baseline ROTEM® analysis was performed using the first ml of shed blood from hemorrhagic shock. The next ROTEM® analysis was performed only at the end of the experiment by withdrawing 1 ml of blood from the arterial line before the animal was euthanized. Due to the limited blood volume available, only EXTEM and FIBTEM assays were performed. Since thromboelastometric values for hamsters have not been published yet our data represent the first reference list.

b) Plasma fibrinogen concentrations and platelet count—Fibrinogen concentrations and platelet count were determined from the first milliliter of shed volume from hemorrhagic shock; after treatment with either fibrinogen concentrate or sham, blood was withdrawn again for the same measurements. Plasma fibrinogen concentrations were

determined using the Clauss method. Platelet counts were measured with the Abbott Cell-Dyn 3500 Hematology Analyzer (GMI Inc., Ramsey, MN).

c) Microhemodynamics—Arteriolar and venular *RBC centerline velocities **(V)*** were measured on-line using the photodiode cross-correlation method (30) (Photo Diode/Velocity Tracker Model 102B, Vista Electronics, San Diego, CA). Measured *V* was corrected according to vessel size to obtain mean RBC velocity (31). Video image-shearing was used to measure *vessel diameter **(D)*** (Image Shearing Monitor, Vista Electronics, San Diego, CA) (32). *Blood flow **(Q)*** was calculated from the measured values as $Q = V \times \pi(D/2)^2$.

Functional Capillary Density (FCD): Capillaries were considered functional if RBCs transit was observed through the capillary segments during a 30 sec period. FCD was tabulated from capillary lengths with RBC transit in an area comprised of 20 successive microscopic fields under 40x magnification. FCD (cm^{-1}) is the total length of RBC-perfused capillaries divided by the surface area. The relative change in FCD from baseline levels, at each time point, is indicative of the extent of capillary perfusion (33). FCD is a key parameter for evaluation of microvascular perfusion and severity of shock.

d) Systemic Parameters—MAP and HR were monitored continuously (MP 150, Biopac System, Santa Barbara, CA). Arterial blood samples taken in heparinized microcapillary tubes (50 μl) were centrifuged to determine hematocrit (HCT). Hemoglobin content (Hb) was determined spectrophotometrically from a single drop of blood (B-Hemoglobin, Hemocue, Stockholm, Sweden).

e) Blood Chemistry—Arterial blood was collected in heparinized glass capillaries (50 μl) from the carotid catheter and immediately analyzed for PaO_2, $PaCO_2$, base excess (BE) and pH (Blood Chemistry Analyzer 248, Bayer, Norwood, MA). The animals have comparatively low PaO_2 and high $PaCO_2$ as a consequence of their adaptation to a fossorial environment (34).

f) Measurement points—Blood chemistry, systemic- and microvascular parameters were measured at baseline ***(BL)***, one hour after shock ***(S)*** and one hour after resuscitation **(*R*)**. ROTEM® parameters were measured at ***BL*** and ***R***.

Statistical analysis

All parameters besides the microhemodynamic variables were normally distributed and are presented as mean ± SD. One way ANOVA was performed between time points of interest within a treatment group. When appropriate, post-hoc analyses were performed with the Dunn multiple comparison test. Microhemodynamic parameters were not normally distributed and thus results are presented using box-whisker plots (median, lower and upper quartiles). All microhemodynamic measurements were compared with baseline levels. A ratio of 1.0 signifies no change from baseline, whereas lower and higher ratios are indicative of changes proportionally lower and higher than baseline. Statistical analysis with the X^2-test showed there was no difference in microhemodynamic parameters between groups at baseline. Similarly, there was no difference in microhemodynamic parameters between

groups after hemorrhagic shock. Therefore data from both groups at these individual time points where pooled allowing for more robust statistical analysis. Statistical comparison of thrombus formation was made with nonparametric methods by use of the Pearson χ^2 with Fisher exact test. Statistical analyses were performed using computer software (Prism 4, Graphpad, San Diego, CA). Results were considered significant when $p < 0.05$.

RESULTS

Microvascular parameters were analyzed in a total of 98 venules and 45 arterioles. Vessels were recruited from 10 animals which, after resuscitation with Hextend received either fibrinogen concentrate (Treatment: *N = 5 animals*) or an equal volume of saline (Sham: *N = 5 animals*). An extra group of 14 animals underwent exactly the same surgical procedures, were randomized for treatment or sham, but were then used for blood withdrawal for platelet and fibrinogen concentration measurements. A total of 24 animals were entered into this study and all tolerated the protocol without visible signs of discomfort.

1. Coagulation measurements

a) Thromboelastometric parameters—Hemorrhage/shock and following resuscitation significantly reduced FIBTEM and EXTEM maximum clot firmness (MCF) compared with ***BL***. Treatment with fibrinogen restored BL MCF levels (Figure 2). Hemorrhage/shock and resuscitation significantly decreased FIBTEM and EXTEM alpha angle compared with ***BL***; fibrinogen treatment restored baseline values. Also clot formation time (CFT) was significantly prolonged if fibrinogen was not substituted after resuscitation with Hextend (Table 1).

b) Plasma fibrinogen concentration and platelet count—Measurements of plasma fibrinogen concentration and platelets were performed in a separate group of animals (*N = 14*) as described above. Hemorrhage/shock significantly reduced plasma fibrinogen concentration. Resuscitation and treatment with fibrinogen restored ***BL*** plasma fibrinogen concentrations. Animals not receiving fibrinogen exhibited significantly lower fibrinogen values after resuscitation compared with ***BL*** and to animals who had received fibrinogen (Table 1).

Platelet count at the end of the experiment was not significantly changed from baseline in both experimental groups (Table 1).

2. Microcirculation

a) Venules—A total of 98 venules (Treatment: *n = 48*; Sham: *n = 50*; diameter range: 25 – 78 μm) were studied. After one hour of shock, venules exhibited a significant vasoconstriction compared with ***BL***. Resuscitation restored venular diameters of the sham group back to baseline levels; venules of the fibrinogen group showed a significant vasodilation compared with shock (Figure 3A). Venular blood flow significantly decreased after hemorrhage/shock; resuscitation significantly increased venular blood flow in both groups (Figure 3C).

In vivo thrombus formation after laser injury: After 5 sec of exposure to laser light animals treated with fibrinogen showed a significantly higher incidence of thrombus formation compared with non treated animals: The success rate of microthrombus formation in irradiated venules was 37 out of 48 (77%) in the fibrinogen group and 9 out of 50 (18%) in the sham group ($p < 0.0001$). Thrombus formation was not detected in vessels beyond the laser irradiation area.

b) Other microvascular parameters

Arterioles: A total of 45 arterioles were studied (Treatment: *n = 22*; Sham: *n = 23*; diameter range, 23 – 77 μm). Hemorrhage resulted in a significant arteriolar vasoconstriction compared with ***BL*** (Figure 3B). After resuscitation arterioles of the sham group showed a significant vasodilation and returned to baseline levels; arteriolar diameters of the fibrinogen group showed a tendency towards vasodilation, however the changes were not significant (Figure 3B). Arteriolar flow was significantly reduced after hemorrhage/shock compared with ***BL*** (Figure 3D); resuscitation significantly increased arteriolar flow in both groups, however baseline conditions were not restored (Figure 3D).

Functional capillary density (FCD): Functional capillary density was significantly reduced during shock compared with ***BL***. After resuscitation FCD was partially restored, but remained significantly different from ***BL***. There was no difference in FCD at any time point between groups.

3. Systemic parameters and blood chemistry

Hemorrhage induced a significant drop in HCT compared with ***BL*** (31.7 ± 4.0% vs. 49.6 ± 1.8% at ***BL***). Resuscitation and treatment with fibrinogen/saline significantly decreased HCT further to 21.8 ± 2.4% (sham) and 22.2 ± 0.8% (treatment). As expected, hemoglobin showed the same trend as HCT: hemorrhage significantly decreased Hb from 14.6 ± 0.6 g/dl at ***BL*** to 9.3 ± 1.0 g/dl. Resuscitation significantly decreased Hb further to 6.6 ± 0.5 g/dl (sham and treatment). The changes in HCT and Hb values were not significantly different between groups.

Following hemorrhage, MAP significantly decreased from 116 ± 7 mmHg to 57 ± 7 mmHg. Resuscitation partially restored MAP (85 ± 9 mmHg for sham and 83 ± 11 mmHg for treatment); however MAP was still significantly lower than at baseline. No statistically significant differences in MAP were found among the two study groups.

Hemorrhage/shock introduced significant changes in acid-base balance: pH significantly decreased from 7.37 ± 0.04 to 7.22 ± 0.06; base excess (BE) significantly decreased from 5.6 ± 3.6 to −7.9 ± 7.0. pH was restored to baseline levels after resuscitation (7.33 ± 0.06 for sham and 7.34 ± 0.03 for the treatment group) while BE remained significantly lower than ***BL*** (1.1 ± 5.0 for sham and −1.0 ± 4.0 for the treatment group). There were no significant differences in pH and BE between the two groups.

DISCUSSION

This study shows that administration of fibrinogen significantly increased the incidence of microthrombus formation after shock/resuscitation with high molecular weight HES (Hextend) compared with untreated animals. These findings are corroborated by thromboelastometric measurements showing that fibrinogen was able to normalize reduced clot firmness following dilutional coagulopathy. Additionally, thrombus formation was only observed in vessels exposed to laser irradiation. In both the fibrinogen and untreated group there was no incidence of spontaneous clot formation. Thus the presence of excess fibrinogen did not lead to random thrombogenesis. It is therefore suggested that at the microvascular level the increased clot strength, measured by thromboelastometry, corresponds to an increased ability to form a thrombus in laser injured vessels.

Current guidelines for the treatment of hemorrhagic shock emphasize damage control resuscitation (DCR), where hypotensive resuscitation, a strategy restricting fluid administration until hemorrhage is controlled, is combined with hemostatic treatment and damage control surgery (35). Hemostatic treatment addresses the issue that rapid volume resuscitation needed to restore perfusion pressure, induces dilutional coagulopathy which might cause additional blood loss and therefore deteriorates the condition of a severely injured person (36–39). In this scenario fibrinogen reaches critically low levels before any other clotting factor, including platelets (40). Substitution of fibrinogen has been shown to diminish blood loss and improve clot firmness in large animal and patient studies (11, 13, 14). Administration of fibrinogen concentrate in patients undergoing radical cystectomy reduced the need for postoperative red blood cell transfusions (13) and could rapidly correct hypofibrinogenemia thereby controlling bleeding (41) during massive obstetric hemorrhage. Despite these findings, in clinical practice the effect of fibrinogen substitution on clot formation *in vivo* is unknown and treatment is mainly based on *in vitro* laboratory parameters. Although thromboelastometry is considered to provide a more functional coagulation analysis, it still lacks the input of physiological circumstances. The data of this study suggest that thromboelastometric measurements are valid indicators for the clotting situation *in vivo*. One has to be cautious however in extrapolating these results to a clinical situation, since this model does not reflect a clinical situation and is not a trauma model.

The technique of visualizing thrombus formation *in vivo* has been primarily used to better understand the interactions between protein and cellular components involved in the clotting cascade (21, 22, 42). The classical doctrine of an intricate cascade of protease complexes resulting in thrombin generation and finally leading to the conversion from fibrinogen to fibrin, derives mainly from *in vitro* observations, lacking many components that under physiological circumstances play an important role in the process of clot formation. Not only are active cellular components such as endothelial cells, platelets, erythrocytes and leukocytes involved in the hemostatic process but also microparticles deriving from platelets and leukocytes. Additionally, physical forces, e.g. shear forces, generated by the flowing blood have a major impact on clot formation, especially on primary platelet adhesion to the vessel wall (43). From a clinical view point, this factor plays an important role since resuscitation fluids increase perfusion pressure and therefore augment shear forces on the vessel wall which might interfere with firm platelet adhesion.

Until now the effect of shear rates on thrombus formation has only been studied *in vitro*. Studies on excised vessels found that at low shear rates platelet aggregation to the vessel wall is highly dependent on fibrinogen (43), while at high shear rates ($\geq 15\ s^{-1}$) platelet adhesion to the vessel wall only occurred in the presence of von Willebrand factor (vWF). Nevertheless, the establishment of a stable thrombus after primary adhesion requires the presence of both fibrinogen and vWF in order to resist the high shear forces produced by dynamic flow conditions (43). These observations underline that fibrinogen plays a dual role, promoting primary endothelial adhesion at first but eventually contributing to the formation of a stable clot. Considering that shear rates in vessels of the hamster window chamber are low ($100 – 400\ s^{-1}$), our *in vivo* findings could be in concordance with these previous results. However, this conclusion is limited because this model does not allow for investigation of high shear rate vessels.

It is important to note, that the dose of fibrinogen concentrate used in this study is very high, compared with doses administered to humans. Comparative physiology studies across species show that slight variations in the fibrinogen molecule affect its function (44, 45). Therefore it is not possible to simply transferring results of this study to humans, especially in regards to the fibrinogen dose. This study was designed to establish an animal model that allows to study coagulation function in the microcirculation and to compare it to a clinical measurement (thromboelastometry). Our results show that this animal model has the potential to becoming useful tool for further in-depth *in vivo* coagulation studies which would include factors such as clot size, stability and formation velocity as a function of time which cannot be ascertained from *in vitro* measurements.

The small blood volume of the animals used in this study limits our study design because all parameters cannot be measured in the same animal. The baseline levels for fibrinogen and platelet counts were established using a different group of animals from those used in the microcirculation and ROTEM® measurements. This is a confounding factor that can lead to a bias in the interpretation of the results especially in a setting of small experimental groups as in our study. The bias is reduced because the platelet count and fibrinogen levels in the separate group are within the range established for these animals (34).

The classical understanding of thrombus formation postulates that platelet adhesion occurs first, leading to a platelet thrombus, followed by stabilization of the primary thrombus through fibrin strands. Interestingly, even though platelet count was equal at the time point of laser injury between treatment and sham group, the incidence of thrombus formation was highly different between groups. This suggests that the presence of fibrinogen is crucial for primary adhesion of platelets to the vessel wall and that platelet thrombus formation and stabilization through fibrin strands are not strictly sequential steps but are more likely simultaneously occurring events.

It in this study dilutional coagulopathy was induced by the use of Hextend, a plasma expander currently used in the United States. In European clinical practice smaller molecular weight starches (e.g. HES 130/0.4) or gelatins are mainly used as colloidal plasma expanders. It might be speculated that the use of HES 130/0.4 or gelatin would not have induced the same degree of coagulopathy as seen in this study.

CONCLUSIONS

This study shows that the hamster window chamber model can be effectively used for analyzing the microvascular events portrayed by thromboelastometric measurements of clotting ability. The visualization of microscopic blood flow and its consequences on local shear stress provides additional data with which to support conclusions derived from thromboelastometry and should aid in devising hemostatic therapy in patients. Ultimately, studies based on this type of animal model should contribute to the understanding of trauma-associated coagulopathy.

Acknowledgments

The ROTEM® Gamma system used in this study was loaned to UCSD by courtesy of Tem Innovations GmbH, Munich, Germany. This study was supported in part by USPHS Bioengineering Research Partnership grant R24-HL064395 (MI), R01-HL062354 (MI) and USAMRAA award W81XWH1120012 (AGT).

Financial support:

This study was supported in part by USPHS Bioengineering Research Partnership grant R24-HL064395 (MI), R01-HL062354 (MI), R01 HL052684 (PC), P01 HL110900 (MI) and USAMRAA award W81XWH1120012 (AGT).

References

1. Davenport R, Manson J, De'Ath H, et al. Functional definition and characterization of acute traumatic coagulopathy. Crit Care Med. 2011; 39(12):2652–2658. [PubMed: 21765358]
2. Fries D, Martini WZ. Role of fibrinogen in trauma-induced coagulopathy. Br J Anaesth. 2010; 105(2):116–121. [PubMed: 20627882]
3. Frith D, Brohi K. The acute coagulopathy of trauma shock: clinical relevance. Surgeon. 2010; 8(3): 159–163. [PubMed: 20400026]
4. Sorensen B, Fries D. Emerging treatment strategies for trauma-induced coagulopathy. Br J Surg. 2012; 99 (Suppl 1):40–50. [PubMed: 22441854]
5. Brazil EV, Coats TJ. Sonoclot coagulation analysis of in-vitro haemodilution with resuscitation solutions. J R Soc Med. 2000; 93(10):507–510. [PubMed: 11064686]
6. Carr ME Jr. Effect of hydroxyethyl starch on the structure of thrombin- and reptilase-induced fibrin gels. J Lab Clin Med. 1986; 108(6):556–561. [PubMed: 2431085]
7. Jamnicki M, Zollinger A, Seifert B, et al. Compromised blood coagulation: an in vitro comparison of hydroxyethyl starch 130/0.4 and hydroxyethyl starch 200/0. 5 using thrombelastography. Anesth Analg. 1998; 87(5):989–993. [PubMed: 9806670]
8. Mardel SN, Saunders FM, Allen H, et al. Reduced quality of clot formation with gelatin-based plasma substitutes. Br J Anaesth. 1998; 80(2):204–207. [PubMed: 9602586]
9. Fries D. Dilutional coagulopathy: development, diagnostic options and management. Hamostaseologie. 2006; 26(3 Suppl 1):S15–19. [PubMed: 16953287]
10. Fries D, Haas T, Klingler A, et al. Efficacy of fibrinogen and prothrombin complex concentrate used to reverse dilutional coagulopathy--a porcine model. Br J Anaesth. 2006; 97(4):460–467. [PubMed: 16885172]
11. Fries D, Innerhofer P, Reif C, et al. The effect of fibrinogen substitution on reversal of dilutional coagulopathy: an in vitro model. Anesth Analg. 2006; 102(2):347–351. [PubMed: 16428520]
12. Fries D, Krismer A, Klingler A, et al. Effect of fibrinogen on reversal of dilutional coagulopathy: a porcine model. Br J Anaesth. 2005; 95(2):172–177. [PubMed: 15923269]
13. Fenger-Eriksen C, Jensen TM, Kristensen BS, et al. Fibrinogen substitution improves whole blood clot firmness after dilution with hydroxyethyl starch in bleeding patients undergoing radical cystectomy: a randomized, placebo-controlled clinical trial. J Thromb Haemost. 2009; 7(5):795–802. [PubMed: 19320829]

14. Fenger-Eriksen C, Lindberg-Larsen M, Christensen AQ, et al. Fibrinogen concentrate substitution therapy in patients with massive haemorrhage and low plasma fibrinogen concentrations. Br J Anaesth. 2008; 101(6):769–773. [PubMed: 18818192]
15. Gofrit ON, Leibovici D, Shapira SC, et al. The trimodal death distribution of trauma victims: military experience from the Lebanon War. Mil Med. 1997; 162(1):24–26. [PubMed: 9002698]
16. Sauaia A, Moore FA, Moore EE, et al. Epidemiology of trauma deaths: a reassessment. J Trauma. 1995; 38(2):185–193. [PubMed: 7869433]
17. Fries D, Innerhofer P, Schobersberger W. Time for changing coagulation management in trauma-related massive bleeding. Curr Opin Anaesthesiol. 2009; 22(2):267–274. [PubMed: 19390253]
18. Levy JH, Szlam F, Tanaka KA, et al. Review article: fibrinogen and hemostasis: a primary hemostatic target for the management of acquired bleeding. Anesth Analg. 2011; 114(2):261–274. [PubMed: 21965371]
19. Schochl H, Nienaber U, Hofer G, et al. Goal-directed coagulation management of major trauma patients using thromboelastometry (ROTEM)-guided administration of fibrinogen concentrate and prothrombin complex concentrate. Crit Care. 2010; 14(2):R55. [PubMed: 20374650]
20. Schochl H, Cotton B, Inaba K, et al. FIBTEM provides early prediction of massive transfusion in trauma. Crit Care. 2011; 15(6):R265. [PubMed: 22078266]
21. Furie B, Furie BC. In vivo thrombus formation. J Thromb Haemost. 2007; 5 (Suppl 1):12–17. [PubMed: 17635703]
22. Furie B, Furie BC. Mechanisms of thrombus formation. N Engl J Med. 2008; 359(9):938–949. [PubMed: 18753650]
23. Cabrales P, Tsai AG, Intaglietta M. Hyperosmotic-hyperoncotic versus hyperosmotic-hyperviscous: small volume resuscitation in hemorrhagic shock. Shock. 2004; 22(5):431–437. [PubMed: 15489635]
24. Kerger H, Saltzman DJ, Menger MD, et al. Systemic and subcutaneous microvascular pO_2 dissociation during 4-h hemorrhagic shock in conscious hamsters. Am J Physiol. 1996; 270(39):H827–H836. [PubMed: 8780176]
25. Colantuoni A, Bertuglia S, Intaglietta M. Quantitation of rhythmic diameter changes in arterial microcirculation. Am J Physiol. 1984; 246:H508–H517. [PubMed: 6720909]
26. Endrich B, Asaishi K, Götz A, et al. Technical report: A new chamber technique for microvascular studies in unanaesthetized hamsters. Res Exp Med. 1980; 177:125–134.
27. Johnson, PC. Flow measurment techniques in the microcirculation. In: Baker, CH.; Nastuk, WL., editors. Microcirc Tech. London: Academic; 1986. p. 149-159.
28. Cabrales P, Tsai AG, Intaglietta M. Resuscitation from hemorrhagic shock with hydroxyethyl starch and coagulation changes. Shock. 2007; 28(4):461–467. [PubMed: 17558350]
29. Luddington RJ. Thrombelastography/thromboelastometry. Clin Lab Haematol. 2005; 27(2):81–90. [PubMed: 15784122]
30. Intaglietta M, Silverman NR, Tompkins WR. Capillary flow velocity measurements in vivo and in situ by television methods. Microvasc Res. 1975; 10:165–179. [PubMed: 1186524]
31. Intaglietta M, Tompkins WR. On-line measurement of microvascular dimensions by television microscopy. J Appl Physiol. 1972; 32(4):546–551. [PubMed: 5026508]
32. Intaglietta M, Tompkins WR. Microvascular measurements by video image shearing and splitting. Microvasc Res. 1973; 5(3):309–312. [PubMed: 4709728]
33. Tsai AG, Friesenecker B, McCarthy M, et al. Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skin fold model. Am J Physiol. 1998; 275:H2170–H2180. [PubMed: 9843817]
34. Van Hoosier, G., Jr; McPherson, CW., editors. Laboratory Hamsters (American College of Laboratory Animal Medicine). American College of Laboratory Animal Medicine Series. 1. Vol. 57. Seattle: Academic Press; 1987.
35. Rossaint R, Bouillon B, Cerny V, et al. Management of bleeding following major trauma: an updated European guideline. Crit Care. 2010; 14(2):R52. [PubMed: 20370902]
36. Burris D, Rhee P, Kaufmann C, et al. Controlled resuscitation for uncontrolled hemorrhagic shock. J Trauma. 1999; 46(2):216–223. [PubMed: 10029024]

37. Owens TM, Watson WC, Prough DS, et al. Limiting initial resuscitation of uncontrolled hemorrhage reduces internal bleeding and subsequent volume requirements. J Trauma. 1995; 39(2):200–207. [PubMed: 7674386]

38. Deakin CD, Hicks IR. AB or ABC: pre-hospital fluid management in major trauma. J Accid Emerg Med. 1994; 11(3):154–157. [PubMed: 7804578]

39. Moore FA, McKinley BA, Moore EE. The next generation in shock resuscitation. Lancet. 2004; 363(9425):1988–1996. [PubMed: 15194260]

40. Singbartl K, Innerhofer P, Radvan J, et al. Hemostasis and hemodilution: a quantitative mathematical guide for clinical practice. Anesth Analg. 2003; 96(4):929–935. [PubMed: 12651636]

41. Bell SF, Rayment R, Collins PW, et al. The use of fibrinogen concentrate to correct hypofibrinogenaemia rapidly during obstetric haemorrhage. Int J Obstet Anesth. 19(2):218–223. [PubMed: 20194010]

42. Falati S, Gross P, Merrill-Skoloff G, et al. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. Nat Med. 2002; 8(10):1175–1181. [PubMed: 12244306]

43. Ruggeri ZM, Dent JA, Saldivar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. Blood. 1999; 94(1):172–178. [PubMed: 10381510]

44. Jirouskova M, Smyth SS, Kudryk B, et al. A hamster antibody to the mouse fibrinogen gamma chain inhibits platelet-fibrinogen interactions and FXIIIa-mediated fibrin cross-linking, and facilitates thrombolysis. Thromb Haemost. 2001; 86(4):1047–1056. [PubMed: 11686323]

45. Murakawa M, Okamura T, Kamura T, et al. Diversity of primary structures of the carboxy-terminal regions of mammalian fibrinogen A alpha-chains. Characterization of the partial nucleotide and deduced amino acid sequences in five mammalian species; rhesus monkey, pig, dog, mouse and Syrian hamster. Thromb Haemost. 1993; 69(4):351–360. [PubMed: 8497848]

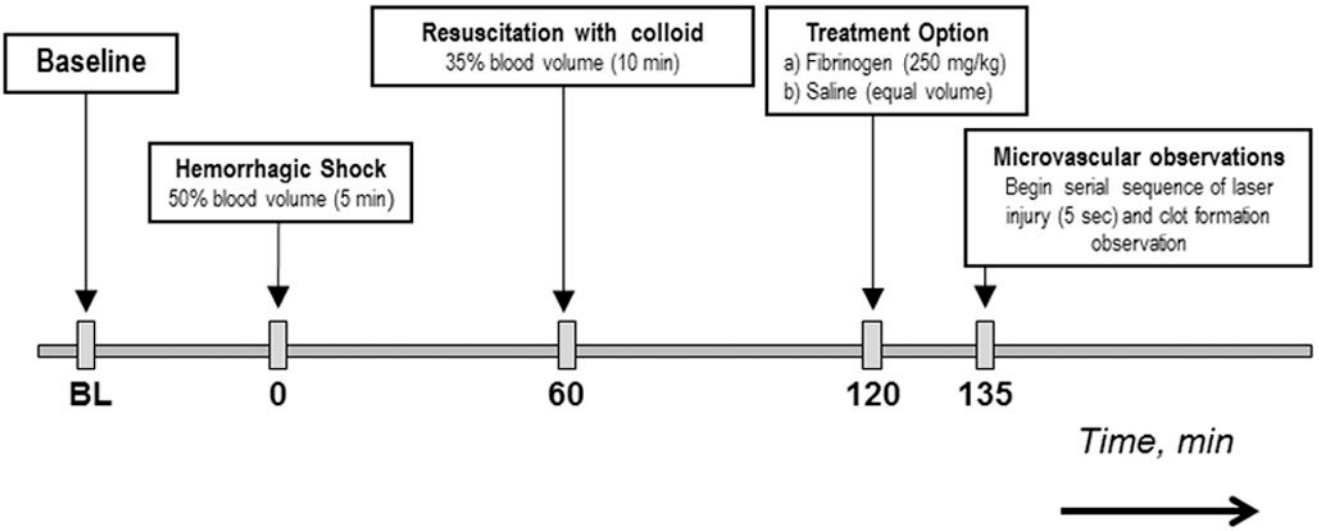

Figure 1.
Experimental protocol.

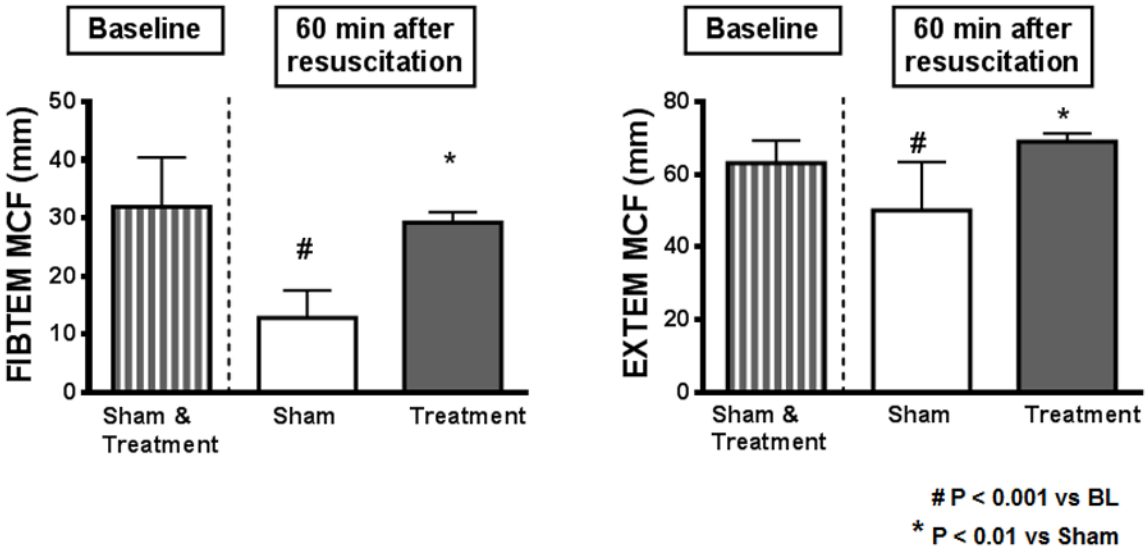

Figure 2. Changes in maximum clot firmness measured by thromboelastometry

Maximum clot firmness (MCF) significantly decreased in FIBTEM and EXTEM after resuscitation with Hextend. Administration of fibrinogen concentrate restored BL levels. Baseline data were pooled for both groups to allow for more robust statistical analysis. #, $p < 0.001$ vs. BL. *, $p < 0.01$ vs. Sham. Bars: Stripped (all animals), white (sham) and grey (treatment).

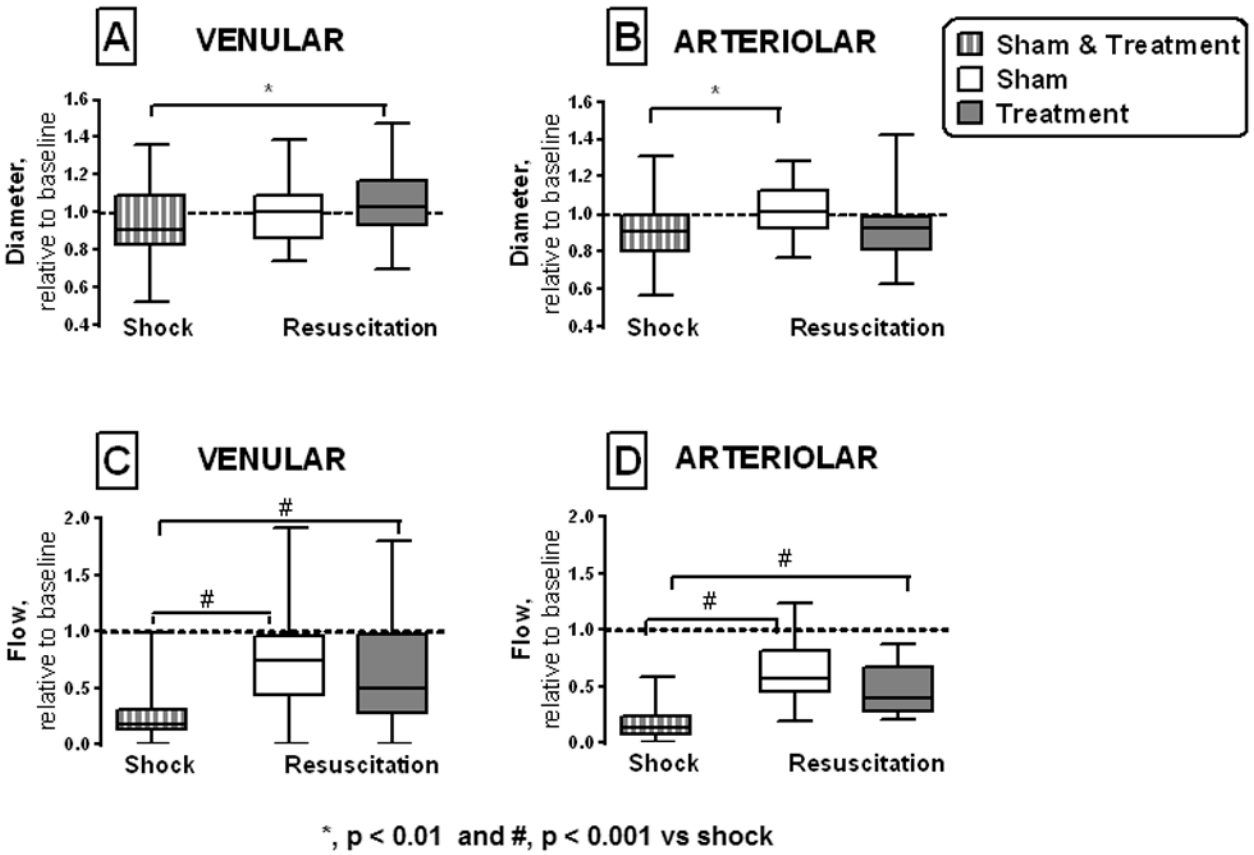

Figure 3. Microhemodynamic changes during shock/resuscitation

Shock resulted in a significant venular and arteriolar vasoconstriction, leading to decreased microvascular blood flow. Resuscitation with HES significantly recovered venular and arteriolar blood flow compared with shock conditions; however, baseline levels were not restored. Data for baseline and shock between the two groups (Sham and treatment) were not statistically different and thus were pooled to allow for more robust statistical analysis. *, $p < 0.01$ vs. shock. #, $p < 0.001$ vs. shock. Bars: Stripped (all animals), white (sham) and grey (treatment).

Table 1

Thromboelastometric parameters, fibrinogen concentration and platelet count at baseline (BL) and after shock/resuscitation.

	BASELINE	After Shock/Resuscitation	
		SHAM	TREATMENT
FIBTEM			
CT (sec)	53 ± 20	59 ± 17	57 ± 35
CFT (sec)	74 ± 34	---[a]	76 ± 33
ALPHA (degree)	80 ± 5	66 ± 3	77 ± 6
MCF (mm)	32 ± 6	13 ± 5	29 ± 2
EXTEM			
CT (sec)	42 ± 8	54 ± 13	48 ± 18
CFT (sec)	55 ± 18	106 ± 51	61 ± 5
ALPHA (degree)	82 ± 2	72 ± 9	79 ± 2
MCF (mm)	63 ± 6	50 ± 13	69 ± 2
Fibrinogen (mg/dl)	440 ± 112	261 ± 62	439 ± 73
Platelet count	350 ± 120	296 ± 71	327 ± 53

[a] Sham animals never reached a CFT value in the FIBTEM test due to their low fibrinogen levels.

* p < 0.01 vs. BL; # p < 0.01 vs. Sham; ** p < 0.001 vs. BL; † p < 0.05 vs. BL.