Continuous enteral protease inhibition as a novel treatment for experimental trauma/hemorrhagic shock

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Acknowledgments:

Department of Defense award W81XWH-17-2-0047 (EBK); 'ShockOmics' grant #602706, 7th Framework Program of the European Union (GWSS, GT); CelSys Shock" Marie Curie International Outgoing Fellowship PIOF-GA-2012-328796, 7th Framework Program of the European Union (FA).

We thank Dr. Rafi Mazor for assistance with the interpretation of the histological images, and Dr. Fernando dos Santos for the critical appraisal of the discussion of the results.

ABSTRACT

Purpose: Trauma and hemorrhagic shock (T/HS) is a major cause of morbidity and mortality. Existing treatment options are largely limited to source control and fluid and blood repletion. Previously, we have shown that enteral protease inhibition improves outcomes in experimental models of T/HS by protecting the gut from malperfusion and ischemia. However, enteral protease inhibition was achieved invasively, by laparotomy and direct injection of tranexamic acid (TXA) into the small intestine. In this study, we tested a minimally invasive method of enteral protease inhibitor infusion in experimental T/HS that can be readily adapted for clinical use.

Methods: Wistar rats were exsanguinated to a mean arterial blood pressure (MABP) of 40 mmHg, with laparotomy to induce trauma. Hypovolemia was maintained for 120 minutes and was followed by reperfusion of shed blood. Animals were monitored for an additional 120 minutes. A modified orogastric multi-lumen tube was developed to enable rapid enteral infusion of a protease inhibitor solution while simultaneously mitigating risk of reflux aspiration into the airways. The catheter was used to deliver TXA (T/HS+TXA) or vehicle (T/HS) continuously into the proximal small intestine, starting 20 minutes into the ischemic period.

Results: Rats treated with enteral protease inhibition (T/HS+TXA) displayed improved outcomes compared to control animals (T/HS), including significantly improved MABP ($p=0.022$) and lactate ($p=0.044$). Mass spectrometry-based analysis of the plasma peptidome after T/HS indicated mitigation of systemic proteolysis in T/HS+TXA.

Conclusion: Minimally invasive, continuous enteral protease inhibitor delivery improves outcomes in T/HS and is readily translatable to the clinical arena.

Keywords: trauma; hemorrhagic shock; enteral infusion; tranexamic acid; protease inhibition; hemodynamics.

1. BACKGROUND

Trauma and hemorrhagic shock (T/HS) is a major cause of morbidity and mortality worldwide [1-3]. Fundamental interventions against T/HS consist of emergent surgical intervention if necessary, and stabilization of hemodynamics by fluid and vasopressor support. Several different therapies have been experimentally tested over the years to treat shock, but none has translated into a clinical modality, with the sole exception of intravenous tranexamic acid (TXA) for early hemorrhage [4-7].

Recently, we outlined the importance of uncontrolled proteolysis as part of the pathophysiology of circulatory shock and as a potential novel therapeutic target. We have previously shown that systemwide proteolysis is increased in experimental hemorrhagic shock compared to healthy animals [8], and these results were independently confirmed by an association found between proteolysis and 28-day in-hospital mortality in septic shock patients [9].

Evidence showing that pancreatic enzymes leak out of the intestine after damage to the mucin/epithelial barrier after intestinal ischemia, resulting in remote organ injury [10-15], provides a possible explanation for the occurrence of systemic, uncontrolled proteolysis in shock. Proteases of pancreatic origin may be active in the circulation and in organs distal to the intestine, directly inducing or mediating (for example by activating proenzymes such as members of the matrix metalloproteinases family) diffuse proteolytic degradation. In particular, both our rat and human studies point to the enhanced role of chymotrypsin-like, trypsin-like and elastase-like enzymes in shock [8,9]. Further, hemodynamic stability, survival and recovery from experimental hemorrhage and other forms of shock are greatly improved following enteral administration of protease inhibitors such as tranexamic acid [16-18] as a possible countermeasure targeting pancreatic proteases in the small intestine, with the aim of inhibiting them before they reach the circulation. Hence, considering the implications of preclinical and clinical data pointing to the occurrence of dysregulated proteolysis and to the effectiveness of protease inhibition in the small intestine, it can be hypothesized that enhanced enzymatic activity is an additional pathophysiological mechanism characterizing circulatory shock, and should be targeted by appropriate treatments aimed to mitigate systemic protein degradation at the source i.e., in the bowel.

However, the main limitation of the previously proposed intervention was the method of delivery, since DeLano's technique required a laparotomy in order to fill the small intestine with inhibitor-carrying solution by serial injections along its entire length [16].

Given that routine laparotomy for shock is not clinically translational, an attractive alternative is to infuse the protease inhibitor enterally by means of a naso-gastric or oro-gastric catheter. Standard enteral feeding tubes (e.g. the Salem Sump™ nasogastric tubes) can serve this purpose, but there are potentially two main technical problems associated with their use: a) enteral feeding is normally carried out at low flow rates, i.e. small volumes over long time intervals, while the emergency treatment of shock requires faster rates to achieve optimal filling of the intestine and inhibition of digestive proteases in minimal time; b) there is no safety system to prevent reflux from the gastrointestinal system into the airways, which is a major issue given the potential for retrograde flow of large volumes of liquid from the stomach into the esophagus.

In this study, we present the results of experiments on trauma/hemorrhagic shock in rats carried out with a twofold goal: 1) to develop and test a clinically viable translational technique for enteral delivery of an enzyme inhibitor in solution; 2) to demonstrate that continuous, minimally invasive enteral protease inhibition improves outcome as assessed by mean arterial pressure (MABP) and mitigates the injury to the gut barrier and systemwide proteolysis.

2. METHODS

2.1. Trauma/hemorrhagic shock (T/HS) experimental model

The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, San Diego (protocol number S15117) and conforms to the Guide for the Care and Use of Laboratory Animals, 8th edition, by the National Institutes of Health (2011). Twelve non-fasted male Wistar rats (400–450 g, Harlan Laboratories, Inc., Indianapolis, IN) were randomly distributed between two groups: untreated (control) trauma/hemorrhagic shock (T/HS, n=6) or trauma/hemorrhagic shock treated with continuous enteral protease inhibition (T/HS+TXA, n=6). Since this study was aimed at developing a translational method for continuous enteral protease inhibition and to assess its impact on systemic proteolysis in shock, no sham group was needed.

After general anesthesia (xylazine, 4 mg/kg, and ketamine, 75 mg/kg, i.m.) the right femoral vein and artery were cannulated for blood withdrawal and intravenous supplemental anesthesia (xylazine, 4 mg/kg; ketamine 7.5 mg/kg), and for continuous monitoring of arterial blood pressure. Body temperature was maintained at 37°C by use of a water-heated animal stage and a heat blanket.

Animals in both groups were allowed approximately 10 minutes for hemodynamic stabilization after induction of anesthesia, vascular line placement and heparinization via the venous line (porcine heparin from Sagent Pharmaceuticals, Schamburg, IL, 1 unit heparin/ml total blood volume, estimated at 6% body weight to prevent blood clotting in the catheters and allow blood withdrawal).

Hemorrhagic shock was induced using a standard fixed-pressure Wiggers model, as described in previous studies [17,18]. Briefly, blood was slowly removed (0.5 ml/min) until a target mean arterial blood pressure of 40 mmHg was achieved. A laparotomy was performed along the midline of the abdomen (about 2-3 inches), between the diaphragm and the lower portion of the peritoneal cavity in order to induce trauma and allow verification of correct placement of a modified orogastric catheter, which was introduced orally for the continuous infusion of protease inhibitor or vehicle into the small intestine. Laparotomy and orogastric tube placement were performed in both groups. T/HS+TXA animals received the protease inhibitor in vehicle (GoLytely®, Braintree Laboratories Inc, Braintree, MA, U.S.A.), while the untreated (T/HS) animals received vehicle only.

MABP was maintained at 40 mmHg for 2 hours. During the hypovolemic period the shed blood was stored at room temperature (22°C) for 2 hours and warmed to 37°C before returning it to the animal. Animals were monitored for an additional 2 hours after blood resuscitation. At the end of this period, animals were euthanized with an intravascular injection of Beuthanasia-D (120 mg/kg, Merck Animal Health). Death was confirmed by loss of pulse and confirmed by bilateral thoracotomy.

2.2. Enteral delivery system and protocol

A modified, multi-lumen orogastric tube consisting of an infusion line and an aspiration line was orally inserted into the esophagus and then through the pyloric sphincter into the duodenum. The aspiration port was positioned at the distal esophagus, in order to intercept and drain potential reflux from the stomach and intestine. The two lines were connected to a multichannel peristaltic pump with independent channels (Reglo

ICC ISM4308, Ismatec, Switzerland) which were set to rotate in opposed directions in order to achieve delivery (through the infusion line) and aspiration (through the aspiration line as needed), respectively.

In previous studies [16], a volume of approximately 15 ml of TXA (127 mM TXA, Cyclokapron, Pfizer) in GoLytely® (0.14 g/ml 0.9% sterile water) was delivered into the small intestine and cecum via sequential injections performed at 1 hour into the hypovolemic period. In the present study, continuous delivery of 17.5 ml of TXA in GoLytely® was started at the time of catheter tip placement into the duodenum and was continued for 2 hours and 30 minutes, at a rate of 0.117 ml/min.

The post-pyloric placement of the tube tip and optimal flow rates were determined empirically through pilot tests performed on a separate set of animals. Placement of the aspiration line was verified post-mortem and confirmed that the low flow rate did not induce suction of the esophageal wall into the catheter lumen nor ischemia in the esophageal wall.

A scheme of the possible geometry of the modified orogastric multi-lumen catheter for enteral infusion is shown in **figure 1** (adapted from [19]).

Fig. 1 Possible geometry of the modified orogastric catheter, adapted from [19]. The inner lumen of the multi-lumen tube is used for infusion, while the outer sections can be used for aspiration of retrograde flow in the esophagus (1); the inner tube is inserted in the stomach (2) and pushed into the small intestine through the pylorus (3). The infusion tube can be equipped with additional sensors (4) to guide the insertion. The solution is infused through the distal opening (5) in the tip, which is placed in the duodenum (6)

2.3. Blood biomarkers

Arterial blood gases, blood lactate (Lactate Plus meter, Nova Biomedical Corporation, Waltham, MA, U.S.A.), and white blood cell (WBC) count (Leuko-TIC® kit, Bioanalytic GmbH, Umkirch, Germany) were measured at three different time points: baseline (BL), at 2-hour into the hypovolemic period (HYPOVOL), and at the end of the two-hour observation window following resuscitation (REPERF).

2.4. Intestinal histology

In order to analyze the integrity of the gut barrier in T/HS and the effects of the enteral protease inhibitor treatment on it, a “whole mount” histological preparation was developed specifically to this end and optimized to minimize the degree of artifactual damage to the tissue induced by non-endogenous causes, such as the treatment and preparation of the sample for analysis. The small intestine was harvested en bloc at

the termination of the experiment, washed and fixed in 10% formalin, and stored at room temperature for whole mount histology. A portion of the distal small intestine just proximal to the cecum, approximately 2 cm in length, was separated for analysis. Prior to analysis, cylindrical specimens (5.0 mm in diameter) were collected from the distal small intestine by means of a disposable biopsy punch (HealthLink, Jacksonville, FL, U.S.A.), rinsed and placed overnight in distilled water to remove residual formalin.

The advantage of this technique, consisting of "whole mounts" of the intestinal wall, is the ability to assess the level of *in vivo* injury over large segments of the gut mucosal layer, rather than views of only single tissue sections and possible *ex-vivo* artifacts arising from tissue processing (e.g., freeze-thaw cycles, vibratome sectioning). This technique thus allows for a more accurate in situ analysis of the intestinal lumen, minimizing potential iatrogenic injury due to manipulation of the tissues.

The mucin-containing mucus layer on the epithelial cells of the small intestine was stained using alcian blue (Alcian Blue Stain, pH 2.5, Diagnostic BioSystems, 6616 Owens Drive, Pleasanton, CA, U.S.A.); the preparation was then rinsed in distilled water and mounted on a microscope slide with the aid of VectaMount AQ Aqueous Mounting Medium (Vector Laboratories, Burlington, CA, U.S.A.).

Precautions were taken to carry out all the steps of the histological analysis under standard conditions in order to allow for comparison of digital images. Bright-field imaging of the specimens was obtained at 4X objective magnification.

2.5. Mass-spectrometry analysis of rat peptidome for assessment of proteolysis

Breakdown of proteins into peptides is a prominent event in shock and was used as a marker of molecular degradation [8,9]. Two venous blood samples of 1.0 mL each were collected in BD Vacutainer® Plus Plastic K2EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.) at baseline before hemorrhage and at the end of experiment before administration of euthanasia from a subset of three rats per group in order to determine the effect of enteral protease inhibition on systemic proteolysis as detected by circulating peptide count and abundance.

A protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Roche) solution was added to the collected blood. Plasma was separated following centrifugation at 1300 rpm for 10 minutes, stored at -80°C and analyzed by mass spectrometry as described in detail in [8,9] to assess circulating peptide count and plasma

protease activity. The total number of circulating peptides at baseline and at the end of the experiment in the two groups was compared as an indicator of the magnitude of ongoing proteolysis in shock.

2.6 Statistical analysis

Results data are reported as mean ± standard deviation. Two-way repeated measures ANOVA was used to assess the effect of the treatment between the two experimental groups at the three different time points, with post-hoc analysis by Tukey's multiple comparisons test. A value of $p<0.05$ was considered to be significant.

3. RESULTS

3.1. Hemodynamics and blood biomarkers

MABP of animals from both the T/HS and T/HS+TXA groups was maintained around 40 mmHg for the duration of the hypovolemic phase. Following blood return, blood pressure transiently recovered in the T/HS group before declining progressively during the 2-hour observation period following resuscitation. In contrast (**figure 2**), blood pressure was restored to baseline levels and maintained throughout the remainder of the experiment in animals who were treated by continuous enteral protease inhibition (T/HS+TXA). The immediate mortality rate in the T/HS (untreated group) was 17% (one animal out six did not complete the experiment and died during the resuscitation period); no animals in the enteral treatment group died.

Fig. 2 Comparison between MABP in the T/HS group and in the T/HS+TXA group (*: p=0.0022). Time [minutes] is from the beginning of the ischemic phase of the experiment

Lactate was significantly increased from basal, pre-hemorrhagic shock values (0.6±0.1 mg/dl) during hypovolemia and after reperfusion in both groups. The enteral TXA-treated group experienced a significantly smaller increase in lactate compared to the T/HS group during the hypovolemic period (3.8±2.1 vs. 9.9±4.6 mg/dl) as well as after reperfusion (2.0±0.5 vs. 5.8±3.6 mg/dl) ($p < 0.01$). pH decreased from baseline (7.38±0.03) significantly more in T/HS compared to T/HS+TXA animals during the hypovolemic period (7.20±0.11 vs. 7.32±0.05) and recovered in both groups at the end of the experiment (7.43±0.11 vs. 7.40±0.05) ($p < 0.0001$ time effect; $p = 0.02$ interaction of treatment and time). Consistent with pH, P_aCO_2 followed a similar trend, decreasing from a baseline of 46±8 mmHg to 34±13 mmHg and 37±7 mmHg at the

end of the hypovolemic period in untreated and in treated animals respectively, and to 20±7 mmHg and 33±6 mmHg at the end of the experiment ($p = 0.0148$ time effect; $p < 0.0001$ treatment effect). Oxygen saturation and P_aO_2 also increased throughout the experiment ($p < 0.0001$ time effect; $p < 0.01$ treatment effect; $p = 0.08$ interaction between time and treatment). WBC count was significantly reduced from baseline in both groups over the three time points ($p = 0.012$) and there were no differences due to treatment at any time point. The plasma biomarkers at different time points are shown in **figure 3**.

Fig. 3 Plasma biomarkers at baseline (BL), at the end of the hypovolemic period (HYPVOL) and at the end of reperfusion (REPERF).
$^{\#}p < 0.01$ time effect; $^{**}p < 0.01$ compared to comparator group at same time point (post-hoc)

3.2. Enteral inhibitor administration and intestinal morphology

The optimal enteral infusion flow rate was experimentally determined based on the concomitant needs to: i) fill the small intestine as quickly as possible; ii) fill the small intestine in its entirety, in order to achieve protection of the gut barrier along its full length, from the duodenum (where the tip of the catheter was placed) to the cecum; iii) minimize retrograde flow into the stomach and avoid the risk of reflux of the infused solution into the esophagus. A satisfactory compromise between flow rate and time of infusion was found and allowed for the small intestine to be infused with the protease inhibitor solution in two hours and thirty minutes at a flow rate equal to 0.117 ml/min (total volume infused was 17.5 ml). The flow rate of the aspiration line was set at 0.01 ml/min in order to prevent possible reflux into the airways, should retrograde flow from the stomach enter the esophagus. A food colorant was added to the solution and used as tracer to verify post-mortem that the solution reached the distal intestine (**figure 4**).

Fig. 4 Small intestine at the end of a test experiment. The presence of food colorant (green) in the distal part of the small intestine and in the cecum demonstrates the ability of the proposed technique to fill the small intestine in its entirety

The distribution of the mucin-containing mucus layer was preserved uniformly across multiple villi in both shocked groups (4x imaging, **figure 5**). The density of the mucin layer was reduced in the untreated animals compared to TXA-treated animals, reflecting potential compromise of the gut barrier in untreated animals, as

well as the mitigation of shock-induced lesions by enteral TXA. The choice of the distal intestine for this histological analysis was aimed at demonstrating the ability of the technique to preserve the intestinal morphology of the entire small intestine, and not only of the region immediately adjacent to the tip of the modified orogastric catheter.

Fig. 5 "Whole Mount" imaging (4x) of the distal small intestine in T/HS+TXA (A) and T/HS (B)

3.3. Proteolysis and enzymatic activity

Analysis of the plasma peptidome after T/HS as an estimation of systemic proteolysis showed that the circulating peptide count (**figure 6**) increased from baseline (102±42) after T/HS in both untreated (220±42) and treated (126±55) animals at the end of the experiment, but the increase was mitigated by treatment with enteral TXA ($p = 0.024$ BL vs. T/HS; $p = 0.027$ BL vs. T/HS+TXA; $p = 0.08$ T/HS vs. T/HS+TXA).

Fig. 6 Estimated proteolysis induced by the T/HS experiment. $p < 0.05$ baseline peptide count compared T/HS group at reperfusion, $p < 0.05$ T/HS vs T/HS+TXA at reperfusion.

4. DISCUSSION

We describe here a novel, clinically translatable therapeutic intervention to efficiently and non-invasively achieve protection of the small intestine with protease inhibitor following experimental trauma/hemorrhagic shock. This innovation allows for continuous enteral delivery of protease inhibitor at relatively high flow rates, by use of a modified naso-/oro-gastric feeding catheter equipped with a safety system to prevent potential reflux into the airways. Continuous enteral infusion of protease inhibitor in a rat model of T/HS resulted in improved hemodynamics and biochemical markers of shock, as well as preserved gut barrier structure and mitigated systemwide proteolysis.

Previous studies from our group demonstrated that enteral TXA has a protective function in the systemic circulation. Injury caused by shock to both the vasculature [17] and the heart [18] is mitigated and adrenergic receptor density in arterial smooth muscle and in the heart is preserved with this treatment and is concordant with the improvements in hemodynamics measured in the present study.

The efficacy of enteral protease inhibition and blood resuscitation in improving outcomes in shock has been previously demonstrated in several models of experimental shock [14]. However, an open problem was that the previously proposed interventions had little translational feasibility. In this paper, we successfully define a new, clinically viable protocol for the continuous enteral infusion of a protease inhibitor-carrying solution. The main advantages of our therapeutic approach can be summarized as follows:

1) oral insertion of a multi-lumen catheter connected to a pumping system can be readily achieved non-invasively, making this intervention clinically feasible;

2) in regard to preservation of the intestinal mucosa, continuous enteral infusion has similar efficacy as compared to the previously reported technique that utilized sequential injections along the length of the small intestine;

3) outcomes were significantly improved by enteral protease inhibition, as shown by the effects of the treatment on the main endpoints of the study, i.e., arterial blood pressure and improved lactate and arterial blood gases;

4) administration of protease inhibitors into the intestinal lumen also had a beneficial impact on systemic proteolysis, as demonstrated by the reduced peptide count and intensity detected in plasma.

Given that gut barrier damage after shock and leakage of digestive enzymes into the systemic circulation can progress in short order and therefore require timely infusion of inhibitor, the small intestine should be protected as quickly as possible. This poses two significant challenges to the development of a safe infusion protocol: optimizing the delivery rate depending on the site of placement of the infusion tube (post- vs. pre-pyloric), and minimizing and preventing retrograde flow from the stomach into the esophagus, which could result in aspiration into the airways and subsequent pneumonia/pneumonitis [20-22]. The latter is still to date one of the most significant risks related to enteral feeding of critically ill patients, which motivates the need for novel technologies to monitor the placement of the enteral feeding tube and prevent and control reflux [23-25]. Thus, the protocol was optimized with the goal of minimizing (and possibly eliminating) the possibility of reflux from the intestine and stomach into the esophagus. Placement of the distal catheter tip in the stomach and the delivery of solution at high flow rates quickly fills the stomach and considerably expands its volume, consistent with the very large compliance of the stomach observed in our experiments. However, the discharge flow rate through the pylorus is considerably slower, and excess stomach dilatation

could result in retrograde flow into the esophagus and potential aspiration. Therefore, for these experiments all enteral flow catheters were placed distal to the pylorus, leaving the reflux orifice of the multi-lumen catheter in the esophagus.

Despite tilting the head of the rats to a 30° angle in order to simulate the posture of a patient in an intensive care unit bed, the anatomy of the region surrounding the pyloric valve favors a slight retrograde flow of fluid from the duodenum into the stomach. However, the flow rate chosen for the study did not result in significant retrograde flow. Faster infusion rates may enhance this retrograde flow, however, and cause a large increase in stomach volume and intragastric pressure, thus increasing risk for reflux into the esophagus. The proximal lumen of the catheter was designed as a safety feature, i.e. a line able to drain the esophagus from any retrograde flow from the stomach and prevent aspiration into the upper airways. Importantly, we did not detect any obstacle to the diffusion of the solution in the intestine, despite the presence of chyme in the bowel and limited peristalsis that accompanies T/HS, and the continuous flow regime was adequate to achieve filling of the small intestine.

The effectiveness of this clinically translatable approach was demonstrated by the improvements shown by the enteral TXA-treated rats in all the main endpoints measured in the study: hemodynamics, arterial blood gases, and lactate. The beneficial impact of enteral protease inhibition can be interpreted as a consequence of improvements in hemodynamics and therefore tissue perfusion. Given that the treatment with enteral TXA was able to restore blood pressure and maintain it during reperfusion, it can be assumed that tissue perfusion was restored too. In the presence of physiological perfusion, we hypothesize that the switch from aerobic to anaerobic metabolism is mitigated (or possibly reversed), thus explaining the tendency to recover values of lactate and blood gases closer to basal levels.

To further demonstrate the efficacy of enteral TXA treatment in T/HS we also investigated the morphology of the intestinal barrier using a technique specifically developed to analyze the integrity of the mucin-containing mucus layer that lines the lumen the intestine and the villi themselves. Our qualitative analysis shows that the delivery of the TXA-carrying solution mitigated the mucosal injury along the entire length of the small intestine, suggesting that filling the small intestine in its entirety with protease inhibitor has a beneficial effect on the preservation of the gut barrier.

No significant differences were detected in WBC count between the two experimental groups at any time point (despite a significant intragroup variation over time in both groups), a relatively non-specific indicator of inflammation. This could be due to leukocyte trapping in the microcirculation that occurs during ischemia regardless of other experimental maneuvers and interventions, while only a subgroup of cells continues to circulate. In fact, it has been reported that shock and reperfusion following ischemia enhance leukocyte trapping and accumulation in tissues such as the coronary capillaries following myocardial ischemia and reperfusion [26], cerebral vasculature [27], liver [28], lung and ileum [29].

Finally, in addition to the hemodynamic and biochemical improvements seen after T/HS with enteral TXA treatment, our preliminary peptidomics analysis suggests that enteral TXA also induced mitigation of the systemwide proteolysis that occurs in both experimental and clinical circulatory shock [6,7]. As limiting systemic proteolysis may be a possible new therapeutic target in shock, the mass spectrometry data represent an important confirmation of the importance of delivering protease inhibitor to the intestine during the acute phase of circulatory shock. Further, the observed improvement in systemic parameters (hemodynamics, plasma peptide abundance), metabolic parameters (blood gases, lactate), and tissue damage to the intestine (considered as a fundamental organ in the generation of proteolysis) achieved by enteral protease administration may have some important pathophysiological implications. As dysregulated proteolysis may be a pathological mechanism initiating and propagating circulatory shock, these results support the hypothesis that targeting enzymatic activity can prevent or mitigate the pathophysiology normally seen in this condition. In particular, inhibiting pancreatic enzymes in the small bowel, allows to prevent enhanced, dysregulated proteolysis at the source by use of concentrations of TXA larger than the ones typically used for intravenous administration.

While this study achieved promising results, it is appreciated that there are some limitations to be addressed in the future in order to improve our protocol and refine it for possible clinical use. The current analysis was carried out with TXA as serine protease inhibitor. As shown previously, other serine protease inhibitors with distinctly different molecular structures also provide protection against development of multi-organ failure in different shock models [14]. All of them need to be delivered in relatively high concentrations to match the high concentrations of digestive enzymes discharged from the pancreas into the small intestine. This evidence highlights the need for the enteral delivery of the protease inhibitors in the presence of intestinal

injury in shock. We are also aware of the limited number of subjects that were analyzed with our peptidomics approach aimed at assessing *in-vivo* proteolysis; of the semi-quantitative findings of the morphological analysis of intestinal tissues, which warrants further validation in the future; and of the lack of a sham group to assess the physiologic status of uninjured intestinal morphology and integrity. However, the study design was aimed to focus specifically on the precise effects of the enteral protease inhibition treatment as compared to untreated T/HS and did not have the ambition to characterize the morphology of the intestine in healthy rats. Finally, the opportunity to replicate our study in large animals should be considered a necessary pre-clinical step before human testing.

5. CONCLUSIONS

The clinical viability and the advantages of the novel catheter system presented in this paper, combined with the efficacy of the treatment as measured by several clinically important quantitative endpoints, suggests that our continuous, minimally invasive enteral treatment represents a potential novel intervention against shock. Dedicated protocols using this approach can be easily implemented not only in intensive care units, surgical suites and emergency departments, but also in ambulances or more austere environments, including the battlefield in the case of military applications.

LIST OF ABBREVIATIONS

T/HS: Trauma/hemorrhagic shock

TXA: Tranexamic acid

T/HS+TXA: Trauma/hemorrhagic shock with enteral tranexamic acid (experimental group)

MABP: Mean arterial blood pressure

HYPVOL: hypovolemia (experimental period following blood withdrawal)

REPERF: reperfusion (experimental period following resuscitation)

WBC: White blood cells

DECLARATIONS

Funding

Department of Defense award W81XWH-17-2-0047 (EBK); 'ShockOmics' grant #602706, 7th Framework Program of the European Union (GWSS, GTW); CelSys Shock" Marie Curie International Outgoing Fellowship PIOF-GA-2012-328796, 7th Framework Program of the European Union (FA).

Conflict interest / Competing interests

FAD and GWSS own stock in Inflammagen Inc., a company that develops new shock treatments.

Ethics approval

The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, San Diego (protocol number S15117) and conforms to the Guide for the Care and Use of Laboratory Animals, 8th edition, by the National Institutes of Health (2011).

Consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed for this study are available from the corresponding author on reasonable request.

Code availability

Not applicable

Authors' contributions

FA: study design; animal experiments; data analysis; histological preparations; conception, design, realization and testing of the multilumen catheter; results discussion and interpretation; manuscript draft.

FAD: animal experiments; conception of the histological preparations; design and realization of the multilumen catheter; manuscript revision and approval.

EM: execution of the peptidomics experiments; peptidomics data analysis, discussion and interpretation; manuscript revision and approval.

HM: histological preparations and analysis; manuscript revision and approval.

GWSS: study design; design of the multilumen catheter; results discussion and interpretation; manuscript revision and approval.

GT: design of the peptidomics experiments; eptidomics data discussion and interpretation; manuscript revision and approval.

EKB: study design; critical appraisal of the clinical translational potential of the continuous enteral infusion method; results discussion and interpretation; manuscript revision and approval.

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FIGURE LEGENDS

Fig. 1 Possible geometry of the modified orogastric catheter, adapted from [19]. The inner lumen of the multilumen tube is used for infusion, while the outer sections can be used for aspiration of retrograde flow in the esophagus (1); the inner tube is inserted in the stomach (2) and pushed into the small intestine through the pylorus (3). The infusion tube can be equipped with additional sensors (4) to guide the insertion. The solution is infused through the distal opening (5) in the tip, which is placed in the duodenum (6)

Fig. 2 Comparison between MABP in the T/HS group and in the T/HS+TXA group (*: p=0.0022).
Time [minutes] is from the beginning of the ischemic phase of the experiment

Fig. 3 Plasma biomarkers at baseline (BL), at the end of the hypovolemic period (HYPVOL) and at the end of reperfusion (REPERF). $^{\#}p < 0.01$ time effect; **$p < 0.01$ compared to comparator group at same time point (post-hoc)

Fig. 4 Small intestine at the end of a test experiment. The presence of food colorant (green) in the distal part of the small intestine and in the cecum demonstrates the ability of the proposed technique to fill the small intestine in its entirety

Fig. 5 "Whole Mount" imaging (4x) of the distal small intestine in T/HS+TXA (A) and T/HS (B)

Fig. 6 Estimated proteolysis induced by the T/HS experiment. $p < 0.05$ baseline peptide count compared T/HS group at reperfusion, $p < 0.05$ T/HS vs T/HS+TXA at reperfusion

Figure 1

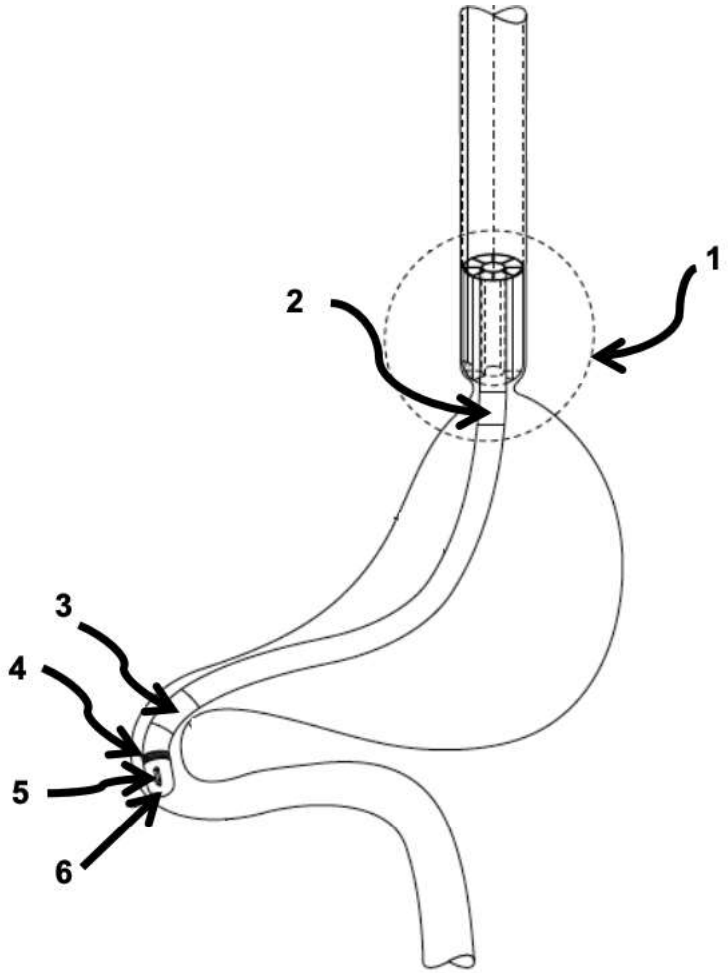


Figure 2

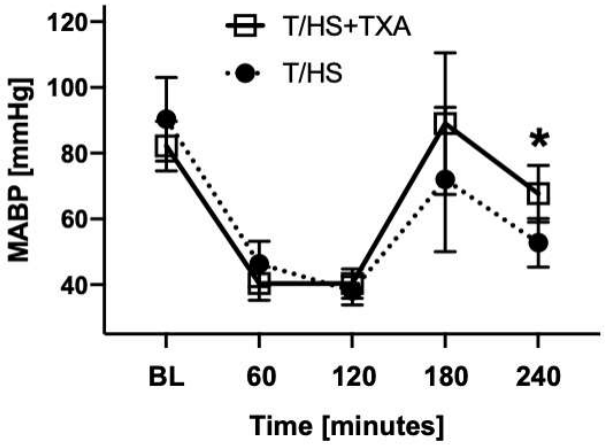

T/HS+TXA
T/HS
MABP [mmHg]
120
100
80
60
40
BL
60
120
180
240
*
Time [minutes]

Figure 3

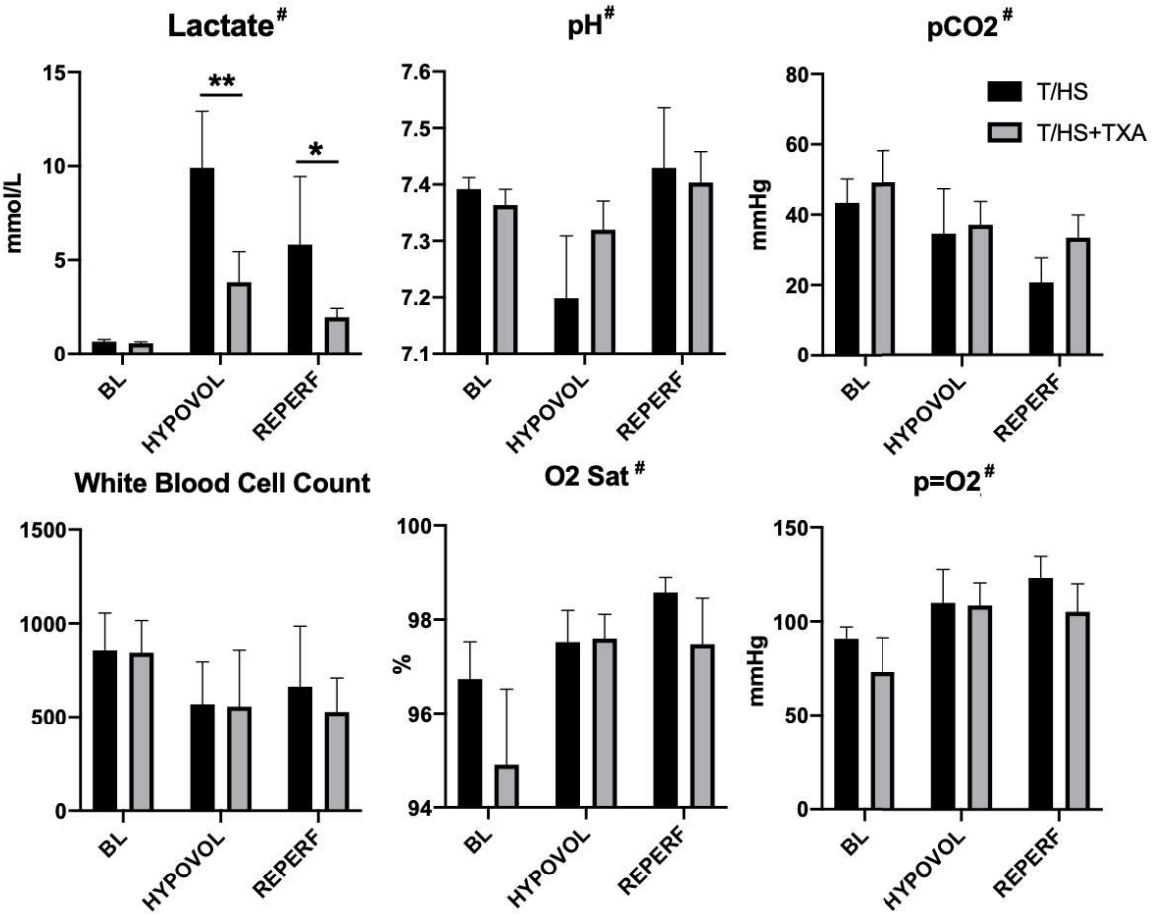
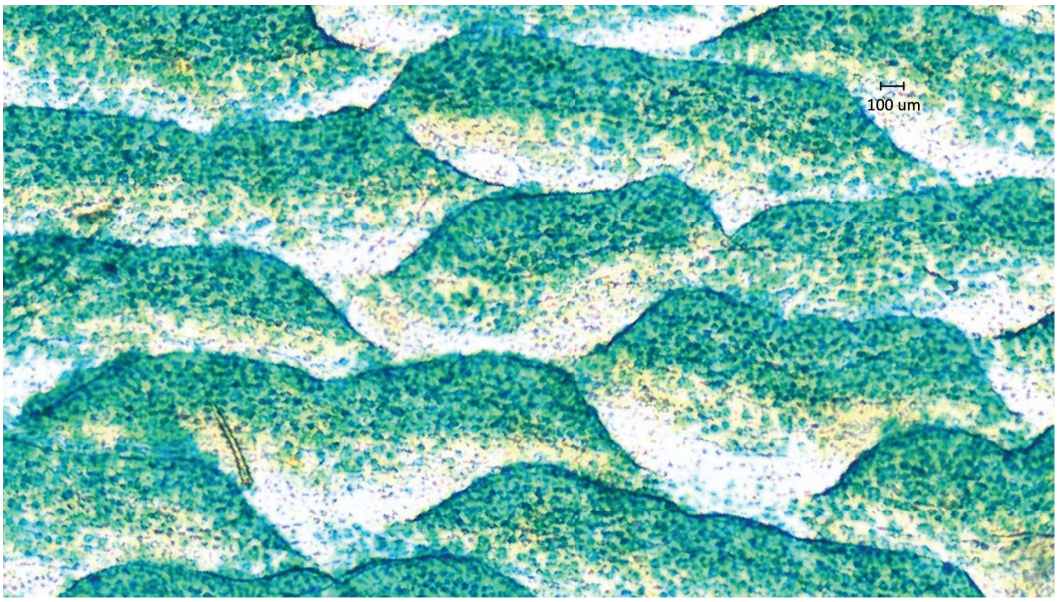

Lactate#
mmol/L
15
10
5
0
**
*
BL
HYPOVOL
REPERF
pH#
7.6
7.5
7.4
7.3
7.2
7.1
pCO2#
mmHg
80
60
40
20
0
T/HS
T/HS+TXA
White Blood Cell Count
1500
1000
500
0
O2 Sat #
%
100
98
96
94
p=O2#
mmHg
150
100
50
0

Figure 4

Click here to access/download;Figure;Fig4.jpg

Figure 5

A



B

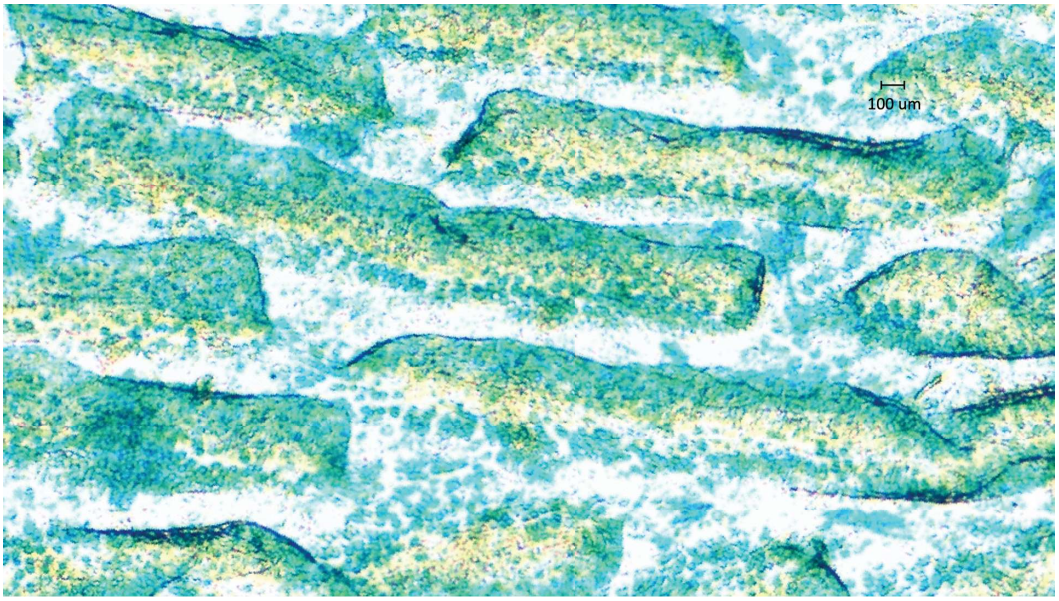
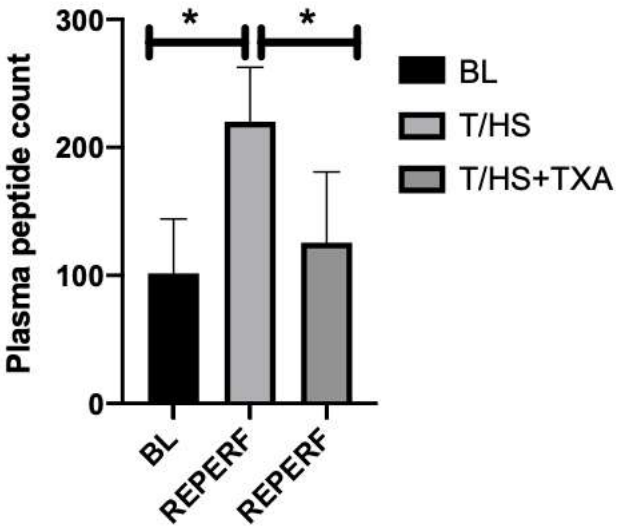


Figure 6


Plasma peptide count
300
200
100
0
*
*
BL
T/HS
T/HS+TXA
BL
REPERF
REPERF