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*Original Article*

# Enteral gabexate mesilate improves volume requirements and autonomic cardiovascular function after experimental trauma/hemorrhagic shock in the absence of blood reperfusion

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**Abstract:** The standard of care for fluid resuscitation of trauma/hemorrhagic shock (T/HS) is the infusion of blood. However, in many instances, blood product transfusion may not be feasible. Consequently, crystalloid solutions may be utilized as temporizing cost-effective resuscitation fluids. In this study, we explored an alternative therapeutic strategy of enteral protease inhibition adjunctive to intravenous Lactated Ringer's (LR) reperfusion after T/HS. Male Wistar rats underwent midline laparotomy (trauma) and an enteral catheter was inserted orally and positioned post-pyloric for the infusion of vehicle (Golytely®) with or without the serine protease inhibitor gabexate mesilate (GM) (n=8/group). Hemorrhagic shock was induced by blood removal to reduce the mean arterial blood pressure (MAP) to 35-40 mmHg for 90 minutes, before resuscitation with LR. Animals treated with enteral GM required significantly less crystalloid volume to achieve hemodynamic stability and displayed improvements in both blood pressure and autonomic function (via increased baroreflex sensitivity to vasopressors, heightened vascular sympathetic modulation, elevated levels of circulating catecholamines, and increased α1-adrenergic receptor density) compared to untreated (control) shocked animals. Resistance arteries isolated from healthy donor animals and perfused with plasma from untreated T/HS animals revealed impaired vascular response to the α1 adrenergic agonist phenylephrine and decreased reactivity to sodium nitroprusside that was preserved in the GM-treated group. These findings suggest that blockade of serine proteases within the intestinal lumen in non-blood resuscitated experimental T/HS preserves and enhances peripheral sympathetic modulation, improving hemodynamics. Enteral infusion of gabexate mesilate may be a new and promising approach to the management of trauma/hemorrhagic shock.

**Keywords:** Trauma/hemorrhagic shock, enteral protease inhibition, gabexate mesilate, autonomic cardiovascular function

## Introduction

Trauma remains a leading cause of death in both civilian and military combat settings in the US and globally, with mortality resulting predominantly from hemorrhagic shock secondary to injury [1, 2]. The current standard of care for treatment of trauma/hemorrhagic shock (T/HS) includes source control and repletion of the vascular space with blood and blood products [3]. However, in low resource and remote environments, blood products may not be readily available. In this case, adjuvant treatments may be required [4].

Trauma-induced hemorrhage leads to a cascade of physiologic responses, primarily from the autonomic nervous system, in an effort to restore hemodynamic stability [5]. The sympathetic nervous system (SNS) responds to blood loss by increasing vascular tone, systemic vascular resistance, and subsequently venous return, in an effort to maintain blood pressure at a level adequate to ensure vital organ perfu-

sion [6]. However, when homeostasis is not achieved, the SNS compensatory mechanisms begin to fail [7, 8]. The decreased vascular activity in response to the marked increase of endogenous and exogenous catecholamines seen in trauma-hemorrhagic shock (T/HS) suggests desensitization [9, 10] in part secondary to a reduction in smooth muscle α1-adrenergic receptor density [11]. In severe T/HS, victims may ultimately become refractory to resuscitation fluids and vasopressors, which then have little to no effect on rescuing and maintaining mean arterial blood pressure (MAP) [12, 13].

In addition to other compensatory mechanisms, the activated SNS in T/HS is inhibitory to the gastrointestinal (GI) tract, decreasing GI mucosal secretion, motility, and blood flow via neurally-controlled vasoconstriction [14, 15]. The weakened integrity of the mucosal barrier eventuates into a semipermeable membrane enabling the leakage of enteral proteases and other low-molecular weight substances into the systemic circulation [16-18]. Once present in the bloodstream, these proteases and their proteolytically-cleaved peptide products may have vasoactive effects, potentially exacerbating the shock condition [19]. In an attempt to mitigate the egress of GI-derived vasoactive mediators systemically and their potential pathophysiological effects in shock, we have recently demonstrated that enteral protease inhibition improves survival in a blood-resuscitated experimental model of T/HS [20]. We hypothesized that enteral administration of protease inhibitors may improve outcomes in non-blood resuscitated experimental T/HS by maintaining hemodynamic stability through improved regulation and control of the autonomic nervous system.

## Material and methods

### *Experimental protocol*

Sixteen male Wister rats (380-440 g, Charles River Laboratories, San Diego, CA) were randomly assigned to receive enteral infusion with either the serine protease inhibitor gabexate mesilate (GM) in vehicle (Golytely®) (GM, n=8) or vehicle alone (Untreated, n=8). Anesthesia was induced in an isolated chamber with 5% isoflurane, followed by nose cone anesthetic delivery at a maintenance level of 1.5% isoflurane in 21% oxygen concentration ($FiO_2$) at a flow rate of 0.8 L/min. The right femoral vein was cannulated for blood withdrawal and intravenous (IV) fluid therapy (PE 50 tubing), and the right femoral artery for monitoring of blood pressure and heart rate (PE 10 tubing). Continuous heart rate and blood pressure were recorded with the Power Lab® data acquisition system using LabChart 7.0 software with a frequency sample of 2 khz/channel (ADInstruments, Dunedin, New Zealand). Partial pressure of arterial oxygen content ($PaO_2$) and carbon dioxide ($PaCO_2$) were monitored regularly to ensure stable respiratory function. Body temperature was maintained at 37°C via a water-heated platform and was monitored throughout the experiment by a rectal probe.

After a short period of vital sign surveillance, a laparotomy was performed (trauma) and a double-lumen enteral catheter was inserted orally post-pyloric so that its tip lay 3 cm into the proximal duodenum. The catheter consists of an inflow tube for solution infusion (PE 50 tubing with a 3 cm Tygon® guide tip, inner diameter of 0.8 mm) and an outflow tube (PE 10 tubing with orifice located in the distal esophagus) to prevent reflux, with both tubes connected to an external peristaltic pump, adapted from Aletti, et al. [20]. All animals received enteral infusion of the carrier solution Golytely® (0.14 g/mL sterile water, 110 μl/min for 150 min) starting 20 minutes into the hypovolemic period. The time-point for starting enteral resuscitation was chosen based on preliminary studies that optimized enteral flow rates necessary to fill the small bowel during the shock and reperfusion periods without appreciable aspiration risk [20]. Blinded to the main investigator, gabexate mesilate (10 mg/kg) was added to the enteral carrier solution in the GM-treated group (GM) but not in the Untreated group.

For ease of blood withdrawal and to prevent clotting of catheters, all animals were intravenously heparinized (100 units/kg). Once the abdominal cavity was closed with suture, the animals were given 20 minutes of hemodynamic stabilization to define baseline. Hemorrhage was induced by removing blood from the femoral vein (0.5 ml/min) until the MAP reached 40 mmHg. MAP was maintained between 35 and 40 mmHg for 90 minutes by removal or return of small aliquots of blood as necessary. After the hypotensive period, IV infusion of Lactated

Ringer's (LR) solution with 5% dextrose was administered (2 ml/min) as reperfusion fluid to achieve blood pressure stability, targeting a MAP of ≥60 mmHg as being commensurate with long-term survival. The animals were monitored for an additional 120 minutes, receiving fluid as necessary to maintain MAP, before being euthanized (B-Euthanasia, 120 mg/kg). Death was confirmed by a complete loss of signal on blood pressure monitor and an adjunctive bilateral thoracotomy.

*Laboratory blood tests*

Blood samples were taken at baseline, the end of the hypotensive period (shock), 30 minutes after reperfusion (r30), and 120 minutes after reperfusion (r120) for measurements of hemoglobin, arterial blood gas, and lactate. Hemoglobin was spectrophotometrically determined (HemoCue HB 201+ System, HemoCue, Brea, CA). Arterial blood gas samples were immediately analyzed upon collection for pH, $PaO_2$, $PCO_2$, $HCO_3$, BE, $O_2$ saturation, and glucose levels (ABL90 FLEX, Radiometer Medical, Denmark). Blood lactate levels were measured using a standard analyzer (Lactate Plus Meter, Nova Biomedical, Waltham, MA). At baseline and r120, whole blood was profiled for metabolic measurements, including albumin, creatinine, globulin, and basic electrolytes (Vetscan VS2, Abaxis, Union City, CA).

*Autonomic assessment*

Temporal series of systolic arterial pressure (SAP) tracings were analyzed in the time domain, obtaining the absolute variability of SAP by standard deviation every 20 minutes. In the frequency domain, the interpolated waves were divided into segments of 512 points, with an overlap of 50%, and were processed by Fast Fourier Transform. A single spectrum was obtained for each segment and the total power was quantified in the frequency band from 0.15 to 0.75 Hz, representing peripheral sympathetic modulation [21]. These data are presented over time, plotting the average of every 20 minutes. Due to the lack of stationarity, periods of fluid removal or infusion were excluded from the blood pressure variability analysis. A comparative spectrum cascade can be found in the supplementary file as Figure S1.

Vascular response to vasoactive drugs and baroreflex sensitivity were analyzed at baseline and r120, after an acute IV infusion of phenylephrine (Phe, 4 μg/kg) or sodium nitroprusside (SNP, 2 μg/kg). The variations in blood pressure caused by these two drugs were used to define vascular responsiveness, and the ratios between the change in heart rate (Δ heart rate) and change in blood pressure (Δ pressure) were used to calculate the reflex bradycardic and tachycardic index, respectively. Catecholamine levels were measured in plasma collected at all four timepoints (baseline, shock, r30 and r120) using colorimetric ELISAs specific for epinephrine and norepinephrine (Novus Biologicals, Centennial, CO).

*Ex-vivo vascular reactivity*

To evaluate systemic vascular function in shock at the vessel level, we measured the vascular response of mesenteric arteries ex vivo as representative resistance vessels. A 3-5 mm arterial third-order segment from mesenteric arteries (approximately 250 μm inner diameter) was cannulated with glass micropipettes in a servo-controlled pressurized myograph chamber (Living Systems Instrumentation, St Albans, VT) containing 6 mL of Krebs solution at 37°C and pH =7.4 and was maintained under constant aeration (95% $O_2$ and 5% $CO_2$). Intraluminal pressure was slowly increased to 140 mmHg using Krebs solution, while the artery was gradually stretched until it reached optimal diameter and tension, and then the pressure was subsequently decreased to 70 mmHg for a 1-hour stabilization period [22]. To determine the role of circulating plasma factors versus non-circulating (tissue) factors in vascular function, two different experiments were performed: 1-Plasma from experimental T/HS animals (n=4 all groups) obtained prior to euthanasia was used to perfuse healthy arteries from non-shocked donors; 2-shocked arteries from both Untreated and GM groups (n=4 all groups) were perfused with Krebs solution rather than plasma. After the stabilization period with Krebs solution, healthy donor arteries were perfused with plasma from Untreated or GM group and allowed to equilibrate for 40 minutes, while shocked arteries from both Untreated and GM groups were perfused with Krebs solution. The maximum contraction capacity was achieved by adding 75 mM potassium chloride (KCl) into

the chamber. Vessels were used if they constricted 50% or more in response to KCl. Concentration response curves (CRCs) were constructed by the cumulative addition of Phe or SNP at concentrations ranging from $10^{-9}$ to $10^{-5}$ M in half-log increments to the mounted arteries. After each set of measurements, the bath chamber was washed with fresh Krebs solution and left to stabilize for 20 minutes. All experiments were performed with intraluminal pressure at 70 mmHg. Arterial contraction and dilation capabilities were expressed as a percentage of the maximum contraction to KCl. The arteries were viewed with a 10X objective equipped with a monochrome video charge-coupled device camera, and their luminal diameter recorded continuously by image capture with a video frame grabber and real-time edge-detection system (VasoTracker 1.0.3) [23]. Krebs solution, or plasma from healthy control animals, was used to establish a baseline curve-response in healthy arteries according to the experiment.

*Immunofluorescence detection of $\alpha_1$-adrenergic receptor on vascular smooth muscle cells*

To evaluate the role of the catecholamine-responsive α1 adrenergic receptor at the cellular level in response to T/HS either in the presence or absence of enteral GM, pulmonary artery smooth muscle cells (SMCs) (RPASMC, Cell Applications, San Diego, CA) were cultured with growth medium (R311-500, Cell Applications, San Diego, CA) in 100 mm dishes at 37°C in 5% $CO_2$. Once 90% confluence was reached, SMCs were dissociated, diluted, and seeded onto 8-well chamber slides. With 60-70% confluence in each well, the SMCs on the slides were serum-starved with basal medium (R310-500, Cell Applications, San Diego, CA) for 16 hours prior to their incubation in fresh basal medium or in diluted plasma (1:5 in basal medium) collected at r120 from experimental T/HS animals (n=4 all groups) for 3 hours at 37°C. SMCs were then washed with Phosphate-buffered saline (PBS) and fixed without permeabilization using 4% paraformaldehyde. Non-specific antigens were blocked with 1% bovine serum albumin (BSA), 22.52 mg/mL glycine in 1X PBS, 0.1% Tween® 20 detergent (PBST) for 45 minutes. The cells were incubated in diluted $\alpha_1$-adrenergic receptor antibody (1:1000 in blocking solution; ab3462, Abcam, Cambridge, MA) overnight at 4°C on a rocking shaker, followed by incubation for 1 hour with diluted fluorescein-labeled secondary antibody (1:500 in blocking solution; FI-1000, Vector Laboratories, Burlingame, CA) in the dark at room temperature. Counterstaining was achieved using a 1:750 dilution of 2 mg/mL DAPI. Three consecutive washing steps were performed for 5 minutes each using PBS after each antibody or stain incubation. After mounting the slide with 80% glycerol in PBS, images were captured at 40x objective magnification. Mean fluorescence intensities of individual cells were measured on ImageJ/Fiji software. Plasma from healthy control animals (n=4) was used to normalize natural $\alpha_1$-adrenergic receptor density.

*Statistical analysis*

All animals were randomly allocated to experimental groups and treatment (GM vs. Untreated) was blinded to investigators. Data were analyzed using GraphPad Prism 6® (GraphPad Software, Inc., San Diego, CA). After testing via the Shapiro-Wilk normality test, the data were subjected to unpaired Student's t test, one-way or two-way analysis of variance (ANOVA) for repeated measurements. When appropriate, post hoc analyses were performed using the Bonferroni multiple comparisons test. Results were considered significant if $P<0.05$. Power analysis and sample size were calculated based on previous results targeting end-blood pressure as primary outcome [20]. In the present study, the primary outcome of interest was the volume of fluid repletion necessary to achieve survivable blood pressure (MAP ≥60 mmHg). All values are expressed as mean ± standard deviation.

*Ethical statement*

The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of University of California, San Diego (protocol number S16062 from 05/17/2017) and conforms to the Guide for the Care and Use of Laboratory Animals, 8th edition, by the United States National Institutes of Health (2011).

## Results

There were no significant differences between experimental groups in body weight and total

**Table 1.** Experimental parameters

| | Untreated | GM | *P*-value |
|---|---|---|---|
| Weight (g) | 408.71±24.96 | 410.36±36.85 | 0.9060 |
| Blood collected (ml) | 13.54±1.55 | 13.38±2.18 | 0.8879 |
| LR infused (ml) | 25.71±2.36 | 16.50±2.51* | 0.0001 |
| Volume infused (%) | 190.54±10.61 | 126.99±31.19* | 0.0001 |
| Lung dry/wet ratio (%) | 17.57±2.25 | 18.30±1.67 | 0.4730 |
| Kidney dry/wet ratio (%) | 20.15±0.92 | 21.58±1.30* | 0.0235 |

Comparison of general experimental parameters between experimental trauma/hemorrhagic shock animals reperfused with Lactated Ringer's solution in the presence (GM) or absence (Untreated) of enteral gabexate mesilate. *P-values of Untreated vs. GM obtained by unpaired t-test.

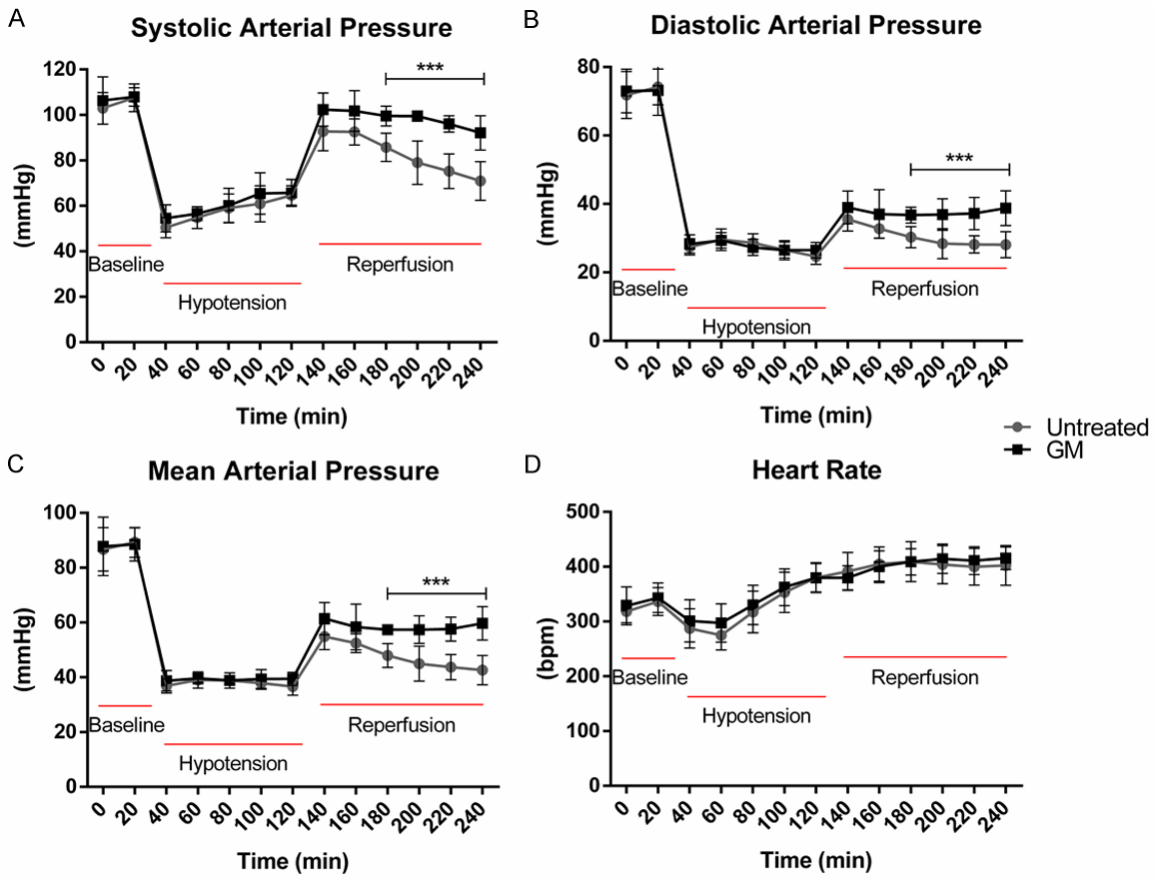


**Figure 1.** Hemodynamics. Time-course of arterial blood pressure (A-systolic, B-diastolic, and C-mean) and heart rate (D) of enteral GM-treated group (GM) and untreated shock group (Untreated). ***P<0.0001 GM vs. Untreated at the same time-point by two-way ANOVA.

amount of blood shed required to induce and maintain hypovolemia (**Table 1**). However, the reperfusion volume required to achieve blood pressure stability was significantly reduced in the GM group compared to the shocked Untreated group. Edema to the kidneys as measured by wet/dry ratios was also significantly reduced in the GM group compared to Untreated; there was no difference in measured lung edema between the groups.

Temporal analysis of biological signals reflects the improved hemodynamics resulting from enteral GM administration (**Figure 1**). The GM group was able to increase and maintain blood pressure, particularly SAP, compared to Untre-

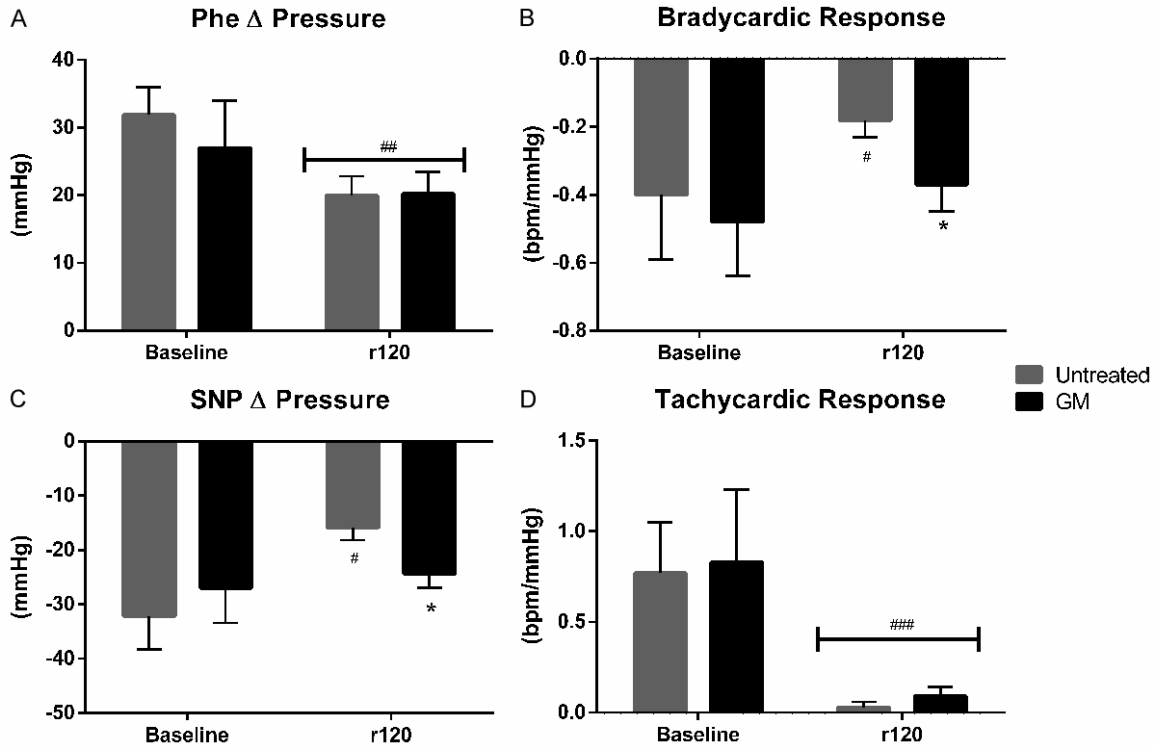


**Figure 2.** Baroreflex sensitivity. Comparison of blood pressure increases at baseline and 120 minutes after reperfusion (r120) in response to in vivo phenylephrine (Phe) infusion (A) and subsequent bradycardic response index mediated by baroreflex (B). Comparison of blood pressure decreases in response to in vivo sodium nitroprusside (SNP) infusion (C) and subsequent tachycardic response index mediated by baroreflex (D) in the enteral GM-treated (GM) and the untreated groups (Untreated). *P<0.01 GM vs. Untreated at the same time-point, #P<0.01, ##P<0.001, and ###P<0.0001, r120 vs. baseline in the same group by two-way ANOVA.

ated upon reperfusion with LR. Heart rate was not different between groups.

Blood pressure response to Phe in vivo was diminished following T/HS with resuscitation in both groups (**Figure 2**). Vasorelaxation due to SNP was also reduced in the Untreated group, but not in the GM animals. The bradycardic response to an acute increase in blood pressure (after Phe challenge) was impaired only in the Untreated group after reperfusion (**Figure 2B**), indicating that enteral GM treatment preserved autonomic response to increases in pressure, improving baroreflex sensitivity to a hypertensive stimulus in T/HS compared to Untreated animals. However, this improved sensitivity was not achieved with acute reduction in blood pressure by SNP, and both groups showed a significant decline in tachycardic reflex in response to this stimulus after reperfusion from T/HS (**Figure 2D**).

In addition to improving systemic blood pressure and vascular reactivity, enteral GM treatment was able to enhance peripheral autonomic modulation after shock. As shown in **Figure 3**, the GM group displayed greater systolic pressure variability as a consequence of the increase in vascular sympathetic modulation. Epinephrine levels in blood plasma after reperfusion were also increased in the GM-treated animals; norepinephrine levels were similar between the groups except for the last timepoint, where there was a reduction of this catecholamine in the GM group.

Isolated artery reactivity was also improved after GM treatment. Plasma from GM group rats was able to preserve normal reactivity in response to phenylephrine (**Figure 4B**) and sodium nitroprusside (**Figure 4D**) in healthy vessels but not in shocked arteries perfused with Krebs solution, regardless of treatment (**Figure 4A** and **4D**).

Immunofluorescence staining of the $\alpha_1$-adrenergic receptor in cultured vascular SMCs revealed a significant decrease in $\alpha_1$-adrenergic

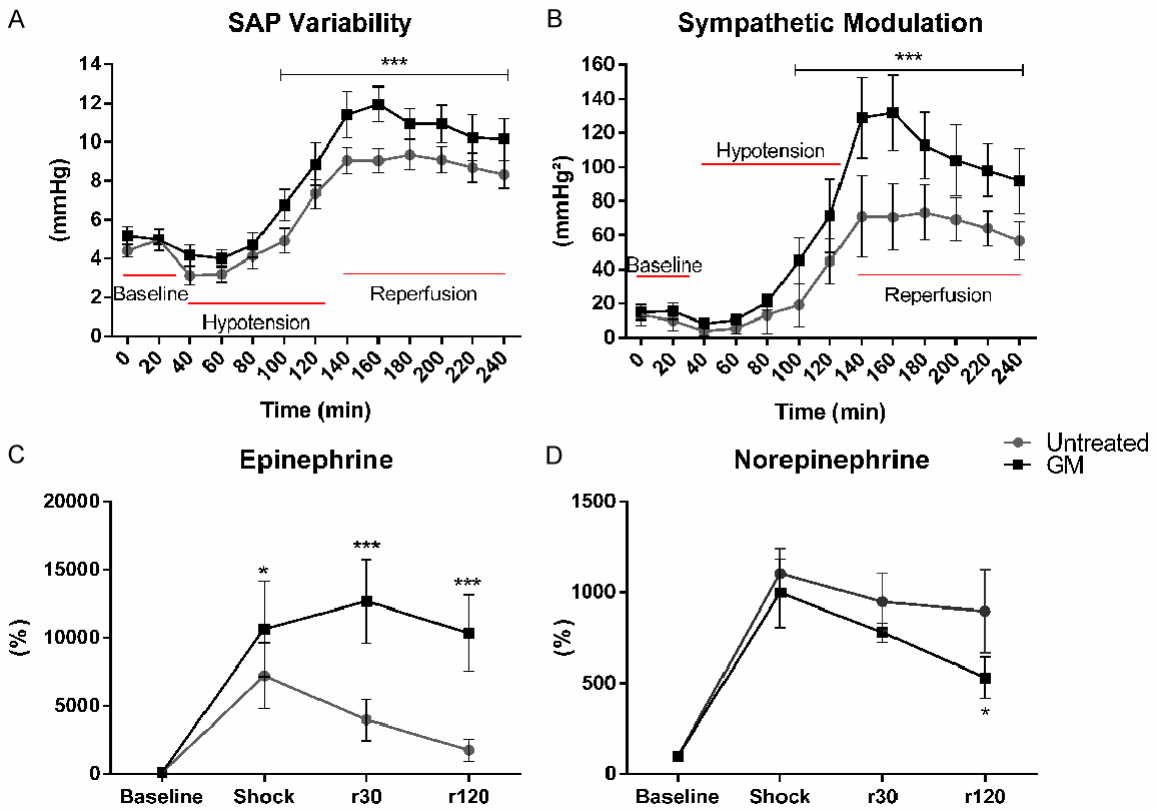


**Figure 3.** Blood pressure variability and catecholamines level. Time course of systolic arterial pressure variability (A), sympathetic modulation (B), and plasma catecholamine levels: epinephrine (C) and norepinephrine (D) at baseline, after the hypotensive period (shock), 30 minutes after reperfusion (r30), and 120 minutes after reperfusion (r120) in enteral GM-treated (GM) and Untreated groups. *P<0.01 and ***P<0.0001 GM vs. Untreated at the same time-point by two-way ANOVA.

receptor density when incubated with Untreated shock plasma as compared to GM shock plasma (**Figure 5**). There was no difference in mean fluorescent intensities between cells exposed to GM shock plasma and plasma obtained from a healthy animal. Images from negative control, non-plasma incubated cells, and non-merged images can be found in supplementary file (Figure S2).

In addition to improvements in autonomic function, enteral GM was also associated with increased circulating levels of albumin, alkaline phosphatase, amylase, total bilirubin, ionized calcium, phosphate, and sodium, which were closer to baseline measurements (Table S1). Blood levels of alanine aminotransferase, urea nitrogen, creatinine, and potassium were elevated in the GM group compared to baseline-these results may be in part secondary to a decrease in overall hemodilution in the GM group. There were no significant changes in arterial blood gas measurements, glucose, lactate, and hemoglobin between the two experimental groups. These data can be found in the supplementary file (Table S2).

## Discussion

Hemorrhagic shock continues to be responsible for considerable mortality in trauma-related patients [4]. Putative pathological mechanisms and treatments for hemorrhagic shock have been extensively studied, with most clinical successes coming from iterative pragmatic damage-control, reliance on blood component reperfusion, and general avoidance of non-blood product reperfusion strategies (TXA being a notable exception) [1, 4, 24].

Given the relevance and the need to discover new treatment strategies for T/HS, we provide a new perspective on the down-stream actions of enteral inhibition of digestive enzymes with

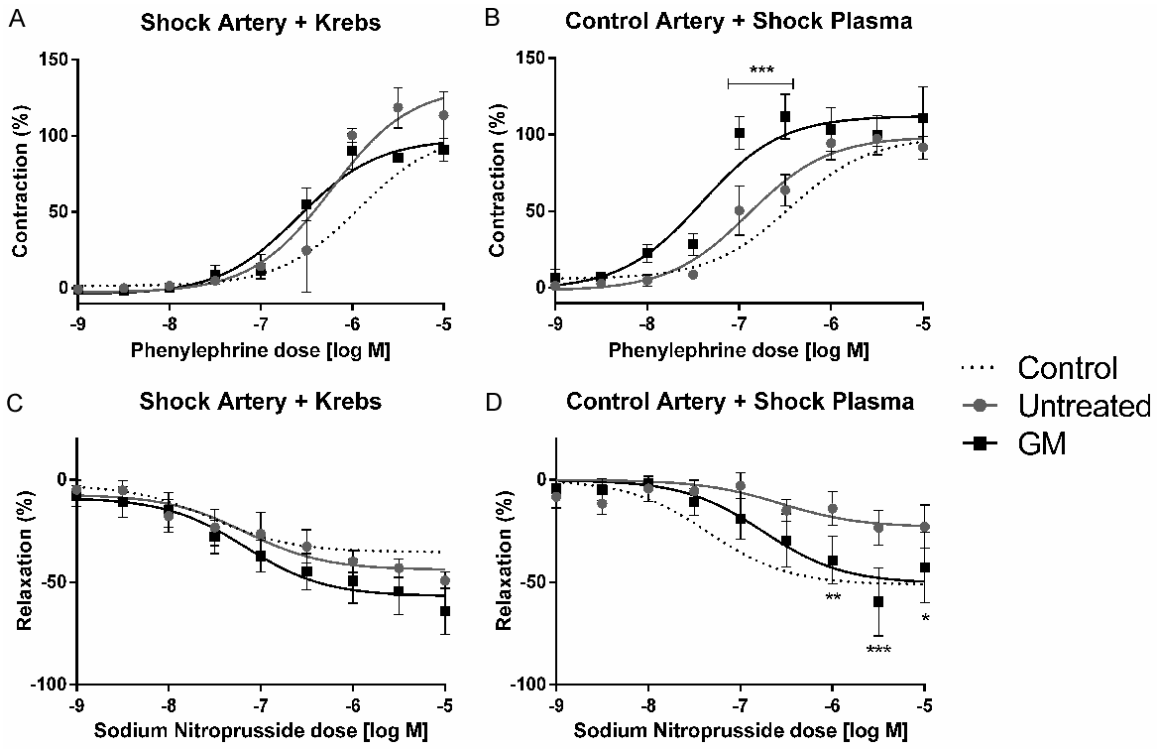


**Figure 4.** Vascular reactivity. Concentration response curves in ex vivo isolated mesenteric artery segments after T/HS. Phenylephrine (Phe) induced contraction of arteries from shocked animals incubated with Krebs solution (A) or healthy donor arteries incubated with plasma from shocked animals (B); Sodium Nitroprusside (SNP) induced relaxation of arteries from shocked animal in Krebs solution (C) and healthy donor arteries incubated with plasma from shocked animals (D) in the enteral GM-treated (GM) and Untreated groups. The dotted lines represent the normal behavior of a healthy vessel in Krebs solution (A and C) or incubated with healthy plasma (B and D) (not included in the statistical analysis). Note that in general, there is an improved vascular response to stimuli in the presence of plasma compared to Krebs solution. *P<0.01, **P<0.001, and ***P<0.0001 GM vs. Untreated at the same concentration by two-way ANOVA.

implications beyond the blockade of proteolytic activity. Our data strongly support the interaction between the serine protease inhibitor gabexate mesilate and the autonomic nervous system. Not only can enteral protease inhibition alter $\alpha_1$-adrenergic receptor density, but it can also impact other components that span the entire autonomic circuit: baroreceptor activity and sensitivity, neurotransmitter concentration in the plasma, peripheral sympathetic modulation, and peripheral resistance. Altogether, these effects resulted in the increase in blood pressure with decreased crystalloid reperfusion requirements in our experiments, resulting in improved outcomes for the enteral GM-treated group in the absence of blood return after T/HS.

Among the less-understood pathologic events occurring in T/HS is intestinal hypoperfusion that may result from low flow states. Sustained periods of hypoperfusion to the small bowel can lead to depletion of the mucus layer, loss of cellular integrity resulting in increased permeability to the systemic circulation, and heightened susceptibility to adverse effects of digestive enzymes and other bowel-derived vasoactive substances [19]. It has previously been demonstrated that animals enterally treated with protease inhibitors after experimental T/HS are able to respond better to blood resuscitation and display improved hemodynamics and survival [11, 25]. In the present study, we delve further into this concept by testing the efficacy of enteral GM treatment in a model of T/HS in the absence of blood resuscitation, and attempt to tease apart the contribution of the autonomic nervous system in this paradigm.

Crystalloid reperfusion in T/HS patients is generally avoided, as its efficacy is poor and it con-

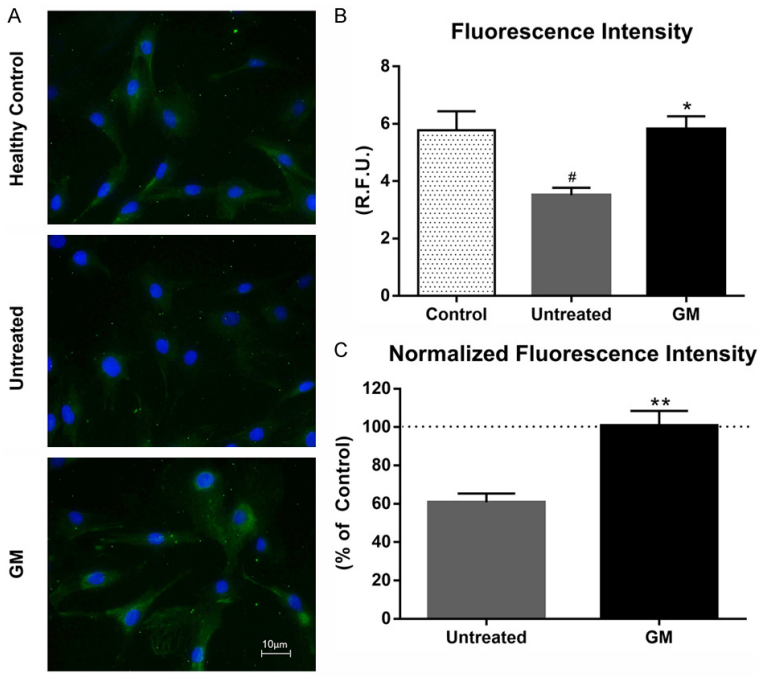


**Figure 5.** Immunofluorescence for α1-adrenergic receptor. Representative images of α1-adrenergic receptor fluorescence on pulmonary artery smooth muscle cells incubated with plasma from healthy animals (Healthy Control), hemorrhagic shock (Untreated), and enteral GM-treated (GM) groups (A). Average fluorescence intensities (B) and fluorescence intensity normalized to Healthy control (C). The dotted line represents 100% intensity in the Healthy Control. #P<0.01 Untreated vs. Healthy Control, *P<0.01 GM vs. Untreated by one-way ANOVA, and **P<0.001 GM vs. Untreated by unpaired t-test.

tributes to a myriad number of iatrogenic issues, particularly dilution of clotting factors [3]. Despite these limitations, in low-resource environments where blood products simply aren't available and crystalloid is much more cost-effective, other resuscitative strategies need to be considered. From these experiments, we found that enteral GM treatment was able to increase MAP by approximately 18 mmHg after reperfusion (**Figure 1**) using only 1.15 times the volume removed (**Table 1**). Thus, enteral GM treatment may be a viable alternative to blood product transfusion in low-resource environments, or as a stop-gap until blood products can be procured.

Of importance when considering the molecular mechanisms involved with enteral GM treatment, improvements in SAP (**Figure 1**) in the GM group to close to baseline levels after resuscitation suggest an improvement in autonomic control in this model. Our first piece of evidence for improved autonomic function centers on the baroreflex. It can be seen from **Figure 2** that both experimental groups had a completely abolished tachycardic reflex (response to SNP), suggesting that the baroreceptors have adapted to the lower blood pressure of shock, since no (additional) cardiac response was detected upon administration of a direct vasodilator. It is important to highlight that even though there was lack of cardiac feedback, the vascular tone was more responsive to SNP in the GM group than in the Untreated group. Regardless, both groups displayed attenuated vasoconstriction in response to Phe when compared to baseline. In the case of Phe, GM improved the baroreflex sensitivity as expressed by the bradycardic response. Those results are very important to the T/HS model, as improved baroreflex function is fundamental for improved long term survival in other cardiovascular diseases, such as heart failure [26].

A second corroborative autonomic enhancement after enteral GM treatment is suggested by results indicating increased vascular sympathetic modulation in this group (**Figure 3**). This parameter does not represent a measurement of sympathetic activity or tone, but rather modulation, meaning the dynamic alterations in vascular tone over time by the SNS. These results, together with the increase in blood pressure variability, suggest that the vascular tone ultimately did not increase (as reflected in the diastolic arterial pressure); however, the vasculature had become more receptive to autonomic command or intervention. By examining the time courses of experimental hemodynamics, it is apparent that the sympathetic modulation curves start to differ in behavior at the 100-minute mark (**Figure 3**), 60 minutes after the start of enteral GM infusion and 30 minutes before reperfusion. This suggests that the effect of GM is independent of the effect of the reperfusion fluid. Increasing peripheral modulation in our model may play a key role in

improving outcomes after T/HS, similar to what happens in diabetic neuropathy (another example of autonomic control depletion), where interventions like renal sympathetic denervation or exercise training help to restore proper modulation and contribute to improved outcomes [27, 28].

Our third piece of evidence suggesting improved autonomic function is the measured catecholamine levels in the GM compared to the Untreated group. During hemorrhage with loss of blood volume and consequently, blood pressure, the body's immediate response to this perturbation is mediated by baroreceptors, which cease their activity to facilitate excitation of the sympathetic efferent system. As a result, several mechanisms are activated in an attempt to increase peripheral resistance, including the release of catecholamines, primarily epinephrine, by the adrenals [6]. Within minutes of stimulation, the plasma concentrations of these neurotransmitters rise to extremely high levels before gradually decreasing over time [29]. In our experiments, GM-treated animals were able to sustain higher plasma concentrations of epinephrine after reperfusion in the GM group compared to the Untreated group (**Figure 3**), again suggesting greater sympathetic control in these animals.

The ex vivo analysis of vascular reactivity (**Figure 4**) demonstrates that the resistance arteries from T/HS animals are restored to normal function and behavior in response to Phe and SNP once placed in a neutral environment (e.g., Krebs solution). Conversely, the vascular response to vasoactive medications in vessels from healthy donor animals incubated with plasma from shocked animals is impaired, but mitigated by treatment with GM, suggesting a direct humoral mechanism upon the vasculature in shock that is inhibited by GM. Once again, the Phe constriction-related curve in the isolated vessels was enhanced in the GM group compared to the Untreated group, providing a fourth piece of direct evidence suggesting improvements in autonomic control in GM-treated animals.

The conservation of $\alpha_1$-adrenergic receptor density on vascular SMCs when incubated with GM shock plasma compared to Untreated shock plasma (**Figure 5**) provides a fifth and focused demonstration of ameliorated autonomic function with enteral GM treatment. Given that the $\alpha_1$-adrenergic receptor is the principal autonomic regulator of vascular constriction in blood vessels, its population density on vascular smooth muscle tissue can directly affect vascular reactivity and tone. Our group has previously shown similar results with TXA-treated T/HS rat plasma [11], suggesting a molecular mechanism occurring at the transmembrane level triggered by proteases or protease-mediated products in concert with classic inflammatory mediators such as toll-like receptor-4 (TLR-4) [30].

## Conclusion

Enteral protease inhibition is a potentially potent adjunct treatment for T/HS based on its ability to improve autonomic function and in particular, vascular reactivity and thus blood pressure. The efficacy of enteral GM after experimental T/HS in the absence of blood product transfusion suggests that such an enteral protease inhibitor approach may be a useful therapeutic option in low-resource environments where blood products are not readily available.

## Limitations

There are some limitations to the current study. The first is that this study, while clinically relevant, is predominantly descriptive in nature, as the complete mechanistic response to enteral protease inhibition in T/HS is not presently well-understood. The use of a relatively short post-reperfusion period for measurement does not allow for measurements of other experimental parameters of potential interest, such as mortality, which can, in the present study, only be inferred by indirect parameters such as blood pressure stability. Finally, we did not, in the present investigation, conduct direct measurements of autonomic tone, making our conclusions as to autonomic nervous system performance very suggestive but not definitive.

Despite showing different autonomic improvements, this paper does not detail mechanisms of actions and/or intend to answer how GM could be contributing directly in the autonomic nervous system. We point out that with this work we are demonstrating for the first time that there is a relationship between them and

how it can be important in the future of T/HS treatment. Our group has been working on new projects in order to identify the possible mechanisms of action, whether by central effect, peripheral, metabolic or even direct enzymatic action. The possibilities so far are numerous, enabling and requiring much more future investigation.

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## Disclosure of conflict of interest

None.

## Abbreviations

GM, gabexate mesilate; T/HS, trauma/hemorrhagic shock; LR, lactated ringer; GI, gastrointestinal; SNS, sympathetic nervous system; IV, intravenous; Phe, phenylephrine; SNP, sodium nitroprusside; MAP, mean arterial pressure; SAP, systolic arterial pressure; KCl, potassium chloride; CRC, concentration response curves; SMC, smooth muscle cells.

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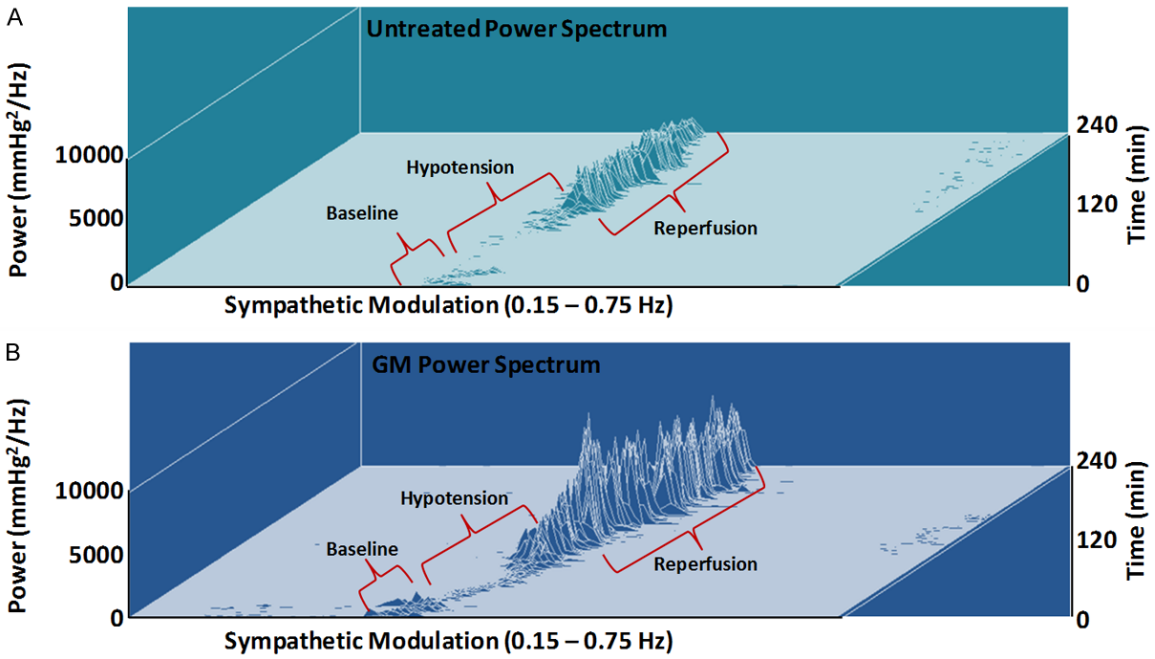


**Figure S1.** Spectral analyses. Representative power spectrum of peripheral sympathetic modulation of one animal from each group. (A) Untreated and (B) enteral gabexate mesilate treatment (GM).

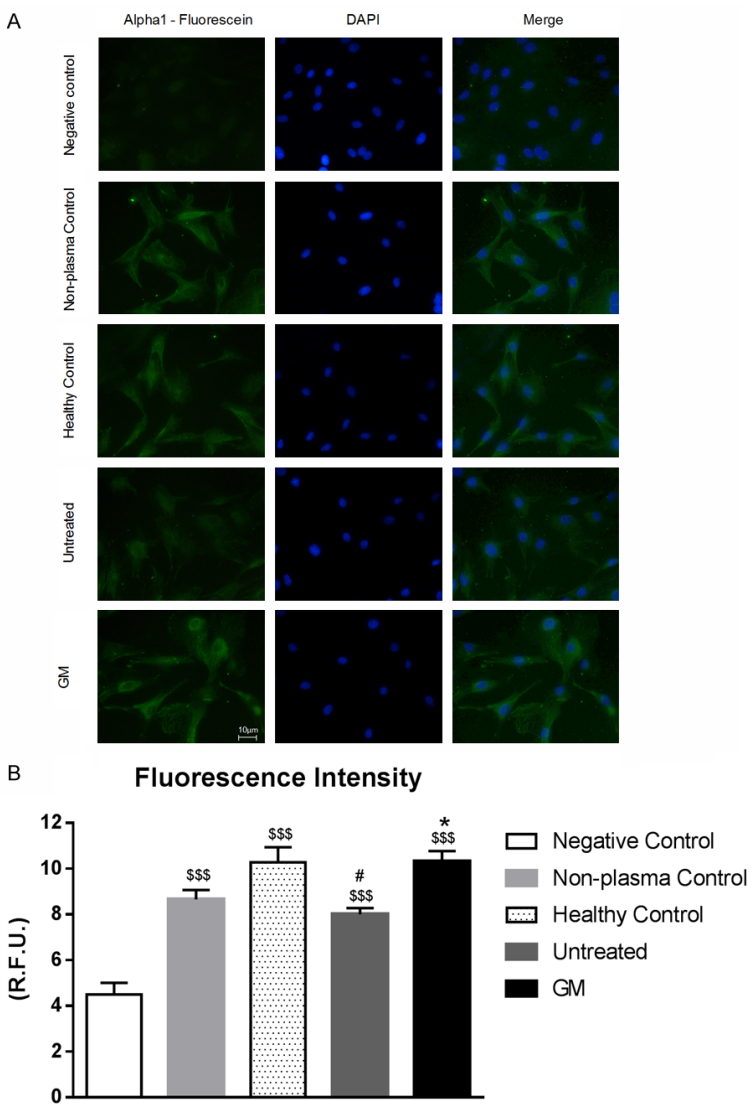


**Figure S2.** Immunofluorescence for α1-adrenergic receptor. Representative images of $\alpha_1$-adrenergic receptor fluorescence on pulmonary artery smooth muscle cells incubated without primary antibody (Negative Control), without plasma (Non-plasma Control), plasma from healthy animals (Healthy Control), hemorrhagic shock (Untreated), and enteral gabexate mesilate-treated (GM) groups (A). Average fluorescence intensities (B). #P<0.01 Untreated vs. Healthy Control, *P<0.01 GM vs. Untreated, and $$$P<0.0001 vs. Negative Control by one-way ANOVA.

**Table S1.** Blood metabolic profile

| | | Untreated | GM | *P*-value |
|---|---|---|---|---|
| WBC (n/μl) | Baseline | 7,214.29±1,284.43 | 5,831.25±1,298.88 | Time <0.0001 |
| | r120 | 2,175.00±883.60# | 3,000.00±1,679.53# | Group =0.5538 |
| ALB (g/dl) | Baseline | 3.96±0.26 | 4.12±0.26 | Time <0.0001 |
| | r120 | 1.46±0.22# | 1.88±0.16*,# | Group =0.0013 |
| ALP (U/l) | Baseline | 190.80±40.34 | 206.80±19.15 | Time <0.0001 |
| | r120 | 126.80±12.50# | 157.75±22.39*,# | Group =0.0155 |
| ALT (U/l) | Baseline | 42.80±7.66 | 51.40±6.80 | Time <0.0001 |
| | r120 | 109.00±36.39# | 491.88±89.06*,# | Group <0.0001 |
| AMY (U/l) | Baseline | 607.80±23.47 | 668.40±39.41 | Time <0.0001 |
| | r120 | 467.00±29.36# | 649.25±43.56*,# | Group <0.0001 |
| TBIL (mg/dl) | Baseline | 0.26±0.05 | 0.20±<0.01* | Time =0.0468 |
| | r120 | 0.20±<0.01# | 0.21±0.04 | Group =0.0468 |
| BUN (mg/dl) | Baseline | 25.60±2.51 | 26.60±1.95 | Time <0.0001 |
| | r120 | 33.40±2.88# | 37.25±1.98*,# | Group =0.0071 |
| $Ca^{2+}$ (mg/dl) | Baseline | 11.20±0.07 | 11.06±0.31 | Time <0.0001 |
| | r120 | 9.04±0.25# | 9.65±0.46*,# | Group =0.0388 |
| PHOS (mg/dl) | Baseline | 9.46±0.99 | 10.00±1.37 | Time =0.1417 |
| | r120 | 8.24±1.16# | 9.66±2.08*,# | Group =0.0678 |
| CRE (mg/dl) | Baseline | 0.50±0.12 | 0.56±0.09 | Time =0.0006 |
| | r120 | 0.52±0.16 | 0.93±0.18*,# | Group <0.0001 |
| $Na^{+}$ (mmol/l) | Baseline | 144.20±1.30 | 141.60±1.14 | Time =0.0709 |
| | r120 | 138.60±2.19 | 144.88±2.10* | Group =0.0059 |
| $K^{+}$ (mmol/l) | Baseline | 4.78±0.48 | 4.94±0.43 | Time <0.0001 |
| | r120 | 5.74±0.67# | 6.38±1.19# | Group =0.1453 |
| TP (g/dl) | Baseline | 5.72±0.34 | 5.54±0.34 | Time <0.0001 |
| | r120 | 2.60±0.25# | 2.98±0.12# | Group =0.3198 |
| GLOB (g/dl) | Baseline | 1.78±0.54 | 1.38±0.22* | Time =0.0003 |
| | r120 | 1.16±0.11# | 1.10±0.15# | Group =0.0441 |

Metabolic profile of shock group (Untreated) and enteral gabexate mesilate-treated group (GM) at baseline, the end of experiment, and 120 minutes after reperfusion (r120). Values found for white blood cells count (WBC), albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBIL), urea nitrogen (BUN), calcium ($Ca^{2+}$), phosphate (PHOS), creatinine (CRE), sodium ($Na^{+}$), potassium ($K^{+}$), total protein (TP), and globulin (GLOB). *P*-value by two-way ANOVA for time and group factors. *P<0.05 GM vs. Untreated at the same time-point; #P<0.05 r120 vs. baseline for the same group.

**Table S2.** Arterial blood analyses

| | | Untreated | GM | *P*-value |
|---|---|---|---|---|
| PH | Baseline | 7.37±0.04 | 7.37±0.06 | Time =0.0063 |
| | Shock | 7.34±0.05# | 7.29±0.09# | Group =0.0722 |
| | r30 | 7.35±0.07 | 7.36±0.08 | Interaction =0.2130 |
| | r120 | 7.38±0.10 | 7.44±0.08# | |
| $PaCO_2$ (mmHg) | Baseline | 51.09±5.71 | 52.63±9.19 | Time <0.0001 |
| | Shock | 34.16±2.17# | 34.54±5.86# | Group =0.0707 |
| | r30 | 37.04±4.80# | 33.19±4.19# | Interaction =0.0601 |
| | r120 | 37.75±3.62# | 30.04±3.01*,# | |
| $PaO_2$ (mmHg) | Baseline | 68.27±9.34 | 69.36±16.53 | Time <0.0001 |
| | Shock | 97.11±15.65# | 100.15±12.16# | Group =0.0008 |
| | r30 | 88.03±14.02# | 104.39±18.63*,# | Interaction =0.0226 |
| | r120 | 83.50±8.01# | 112.30±13.69*,# | |
| $HCO_3$ (mmol/l) | Baseline | 26.81±1.34 | 27.18±1.76 | Time <0.0001 |
| | Shock | 19.04±2.37# | 17.16±2.75# | Group =0.2598 |
| | r30 | 20.86±3.60# | 19.48±2.92# | Interaction =0.7824 |
| | r120 | 22.45±4.18# | 21.67±5.13# | |
| BE (B) | Baseline | 4.37±1.55 | 4.93±2.21 | Time <0.0001 |
| | Shock | -7.26±3.27# | -9.85±3.83# | Group =0.3504 |
| | r30 | -4.91±4.87# | -6.75±3.92# | Interaction =0.6604 |
| | r120 | -2.65±5.57# | -3.51±5.72# | |
| $O_2$ sat (%) | Baseline | 91.26±6.93 | 90.09±15.35 | Time <0.0001 |
| | Shock | 93.19±2.62 | 92.75±3.91 | Group =0.0200 |
| | r30 | 93.56±4.76 | 96.14±5.58# | Interaction =0.3589 |
| | r120 | 93.93±4.66 | 99.47±2.10*,# | |
| Hemoglobin (g/dl) | Baseline | 13.79±1.26 | 13.61±0.98 | Time <0.0001 |
| | Shock | 8.64±1.39# | 8.53±2.19# | Group =0.6640 |
| | r30 | 6.84±1.07# | 7.04±0.86# | Interaction =0.7871 |
| | r120 | 5.75±0.91# | 6.39±0.77# | |
| Lactate (mmol/l) | Baseline | 0.90±0.20 | 0.98±0.34 | Time <0.0001 |
| | Shock | 7.94±1.80# | 10.46±3.11# | Group =0.6924 |
| | r30 | 12.51±2.84# | 12.69±2.10# | Interaction =0.0872 |
| | r120 | 11.58±3.27# | 9.74±2.82# | |
| Glucose (mg/dl) | Baseline | 242.71±58.71 | 305.86±84.29 | Time <0.0001 |
| | Shock | 383.86±152.98 | 445.00±134.12 | Group =0.5039 |
| | r30 | 565.29±140.40# | 545.86±118.43# | Interaction =0.0139 |
| | r120 | 504.67±108.66# | 320.25±120.00 | |

Arterial blood gas profile of shock group (Untreated) and enteral gabexate mesilate-treated group (GM) at baseline, the end of hypotension period (shock), 30 minutes after reperfusion (r30), and 120 minutes after reperfusion (r120). Values for potential of hydrogen (PH), partial pressure of carbon dioxide ($PaCO_2$), partial pressure of oxygen ($PaO_2$), bicarbonate ($HCO_3$), base excess (BE), oxygen saturation ($O_2$ sat), hemoglobin, lactate, and glucose. *P*-value by two-way ANOVA for time, group, and interaction factors. *P<0.05 GM vs. Untreated at the same time-point; #P<0.05 vs. baseline for the same group.