

TITLE:

Serum Diamine Oxidase as a Hemorrhagic Shock Biomarker in a Rabbit Model

ABSTRACT:

**BACKGROUND:** In prolonged hemorrhagic shock, reductions in intestinal mucosal blood perfusion lead to mucosal barrier damage and systemic inflammation. Gastrointestinal failure in critically ill patients has a poor prognosis, so early assessment of mucosal barrier injury in shock patients is clinically relevant. Unfortunately, there is no serum marker that can accurately assess intestinal ischemia-reperfusion injury. **OBJECTIVE:** The aim of this study was to assess if serum diamine oxidase levels can reflect intestinal mucosal injury subsequent to prolonged hemorrhagic shock. **METHODS:** Thirty New Zealand white rabbits were divided into three groups: a control group, a medium blood pressure (BP) group (exsanguinated to a shock BP of 50 to 41 mm Hg), and a low BP group (exsanguinated to a shock blood pressure of 40 to 31 mm Hg), in which the shock BP was sustained for 180 min prior to fluid resuscitation. **RESULTS:** The severity of hemorrhagic shock in the low BP group was significantly greater than that of the medium BP group according to the post-resuscitation BP, serum tumor necrosis factor (TNF)- $\alpha$ , and arterial lactate. Intestinal damage was significantly more severe in the low BP group according to Chiu's scoring, claudin-1, intercellular adhesion molecule (ICAM)-1, and myeloperoxidase expression. Serum diamine oxidase was significantly increased in the low BP group compared to the medium BP and control groups and was negatively correlated with shock BP. **CONCLUSION:** Serum diamine oxidase can be used as a serological marker in evaluating intestinal injury and shows promise as an indicator of hemorrhagic shock severity.

Introduction:

In the battlefield or remote areas, prolonged hemorrhagic shock often results in multiple organ failure (MOF) with a mortality rate of 24% [1]–[4]. The gut is considered to be the “motor” driving MOF [5]– because hemorrhagic shock reduces intestinal mucosal blood perfusion that leads to intestinal mucosal barrier damage, intestinal barrier dysfunction, ectopic flora, endotoxemia, systemic inflammation, and finally MOF [8]–[13]. Clinical studies have revealed that traumatic hemorrhagic shock patients with MOF display significantly higher levels of inflammatory cytokines (e.g., tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1, IL-6) than those without MOF [14], and animal experiments have shown that severe system inflammation is strongly related with prolonged hemorrhagic shock [15].

The timing of resuscitation and the severity of hemorrhagic shock are critical factors affecting intestinal damage. Leukocyte activation and resulting oxidative stress occurs three to six hours after traumatic hemorrhagic shock and is an important factor underlying mucosal barrier damage [16]–[19]. Activated leukocytes, which adhere to vascular endothelial cells via immunoglobulin and intercellular adhesion molecule (ICAM)-1, release peroxides that destroy the intestinal mucosal barrier [20]–[23]. As this gastrointestinal failure in critically ill patients has a poor prognosis [24]–[26], early assessment of mucosal barrier injury in shock patients is clinically relevant.

Unfortunately, there is no serological marker that can accurately assess intestinal ischemia-reperfusion injury. Some studies have found that serum diamine oxidase (DAO) levels are significantly increased after acute mesenteric ischemia [27]–[30], but it is unclear whether serum DAO levels can function as a serological marker in evaluating the severity of intestinal injury induced by hemorrhagic shock.

In this study, a hemorrhagic shock model was constructed with New Zealand rabbits in which two different levels of shock blood pressures (BPs) were maintained for 180 minutes in order to simulate two degrees of prolonged hemorrhagic shock. We hypothesized that prolonged hemorrhagic shock would cause obvious intestinal damage and that serum DAO can be used as an indicator to reflect the differing severities of intestinal ischemia-reperfusion injury accompanying the two degrees of prolonged hemorrhagic shock.

Animal Subjects :: Materials and Methods:

All the following procedures were strictly consistent with 3R principles, while animal care and housing procedures were in compliance with Chinese regulatory requirements. The protocol of this study was approved prior to implementation by the Ethics Committee of Third Military Medical University, and all procedures were in accordance with the National Institutes of Health Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals). Special care

was taken to minimize number of and suffering of animals. Thirty male New Zealand white rabbits (weighing 2.4 to 2.6 kg, aged 6 to 8 months) were purchased from animal facility at Third Military Medical University (Chongqing, China). The rabbits were housed in individual cages and received humane care according to the Principles of Laboratory Animal Care. The laboratory temperature was maintained at  $24\pm 1^{\circ}\text{C}$ .

#### Experimental Protocol :: Materials and Methods:

The 30 rabbits were randomly divided into three groups based on their shock BP levels: the control group, the medium BP group (50 to 41 mm Hg), and the low BP group (40 to 31 mm Hg). All animals were fasted overnight and had free access to water before the experiment. Then, the rabbits were anesthetized by administration of 30 mg/kg 3% pentobarbital, and 20-gauge venous indwelling needles were inserted into the right and left femoral arteries to measure BP and to withdraw arterial blood samples, respectively. A central venous pressure catheter (7.5 Fr.; Baxter Healthcare, Deerfield, IL, USA) was positioned in the right atrium via the right jugular vein for the collection of venous blood samples. The animals were allowed to stabilize for 30 min before induction of hemorrhagic shock.

After a baseline BP was obtained, BP-controlled hemorrhagic shock was induced. The withdrawn blood was collected and heparinized with 10 units/ml heparin. BP was continually monitored and recorded every 2 min. We first used a 180-min prolonged hemorrhagic shock animal model based on the timing of neutrophil activation. The time points corresponding to the initiation of bleeding, the end of prolonged hemorrhagic shock, and 120 min after resuscitation were marked T1, T2, and T3, respectively. Because of autologous transfusion, the shock period from T1 to T2 was separated into two phases. During the first phase, blood was withdrawn at a fast rate of 1 ml/kg/min until the BP reached the target pressure. During the second phase, blood withdrawal or reperfusion was cautiously performed at a slow rate of 0.5 to 1 ml/unit time to stabilize the shock BP. Resuscitation began with equal volumes of heparinized blood and Ringer's solution at T2 at a rate of 2 ml/kg/min. The experiment was concluded at T3 by euthanizing the rabbits through an overdosed intravenous injection of phenobarbital.

#### Specimen Preparation :: Materials and Methods:

The 1-ml venous blood samples were drawn at T1, T2, and T3 with simultaneous infusion of an equal volume of Ringer's solution. The blood samples were centrifuged at 3500 rpm for 15 min at  $4^{\circ}\text{C}$ . After waiting 30 minutes for coagulation, the supernatant was separated and stored at  $-80^{\circ}\text{C}$  for subsequent measurement of DAO, lactate, and TNF- $\alpha$ . Then, a 6.0 to 7.0-cm segment of intestine was resected 10 cm away from the terminal ileum (ileocecal valve) and divided into two segments. One segment was fixed using formalin and embedded in paraffin; the other section was washed with cold saline, dried with blotting paper, and preserved at  $-80^{\circ}\text{C}$ .

#### Scoring Intestinal Mucosal Injury :: Materials and Methods:

The embedded segments of small intestine from all three groups were stained with hematoxylin-eosin. The severity of injury was evaluated by two independent, blinded pathologists using Chiu's scoring method [31]. Briefly, mucosal damage was scored from 0 to 5 as follows: 0, normal mucosal villi; 1, development of sub-epithelial Gruenhagen space at the apex of the villus; 2, extension of the sub-epithelial space with moderate lifting of the epithelial layer from the lamina propria; 3, massive epithelial lifting down the sides of villi, possibly with a few denuded tips; 4, denuded villi with the lamina propria and dilated capillaries exposed, possibly with increased cellularity of the lamina propria; and 5, digestion and disintegration of the lamina propria, hemorrhage, and ulceration.

#### Intestinal Claudin-1 and ICAM-1 :: Materials and Methods:

The embedded intestine was cut into 6- $\mu\text{m}$  sections using a cryostat (LEICA CM 1800, Leica Co., Germany). The sections were further fixed with 4% paraformaldehyde at room temperature for 10 min and washed with PBS. The samples were then immunohistochemically stained with goat polyclonal antibodies against rabbit claudin-1 or rabbit ICAM-1 (diluted 1:50). The secondary antibody (rhodamine-labeled rabbit anti-goat IgG diluted 1:200) was added and incubated at  $37^{\circ}\text{C}$  for 30 min followed by washes with PBS and incubation with Hoechst 33342 (2  $\mu\text{g}/\text{ml}$ ) at room temperature for 3 min. After washes with PBS, the specimen was sealed and observed using a laser confocal microscope. Fluorescence intensity was detected at an excitation wavelength of 543 nm for R-phycoerythrin and 405 nm for Hoechst. Five fields of view (1.6 $\times$ 104  $\mu\text{m}^2$  each) in the rabbit intestinal mucosa were evaluated for each condition. Fluorescence intensities of claudin-1

or ICAM-1 were estimated by Image Pro Plus software and expressed as an average proportion of positive areas per one field of view ( $1.6 \times 10^4 \mu\text{m}^2$ ).

#### Intestinal Myeloperoxidase Activity ::: Materials and Methods:

Myeloperoxidase (MPO) activity in homogenates of the whole intestine (proximal jejunum) was determined as previously described [32]: "The tissues were washed and homogenized using Tekmar tissue homogenizer (Brinkman, Cincinnati, OH) for 20 secs on ice in potassium phosphate buffer (pH, 7.4) containing 2 mM PMSF. The homogenate was centrifuged at 10,000 rpm for 30 mins at 4°C (39.2°F). The pellet (90% of the total myeloperoxidase [MPO] activity), was homogenized in 10 volumes of ice-cold 50 mM potassium phosphate buffer (pH, 6.0) containing 0.5% hexadecyltrimethyl-ammonium bromide and 10 mM EDTA. MPO activity was determined by using a modification of the method (22) in which the enzyme catalyzes the oxidation of 3,3',5,5'-tetramethylbenzidine by hydrogen peroxide. An aliquot of standard recombinant human MPO (0–0.125 units) or samples homogenate was added to 0.5 mL MPO cocktail (80 mM potassium phosphate buffer; pH, 5.4; 0.5% (w/v) hexadecyltrimethyl-ammonium bromide; and 1.6 mM 3,3',5,5'-tetramethylbenzidine). The mixtures of samples with the MPO cocktail were placed at 37°C (98.6°F), and the reaction was started by addition of 0.3 mM hydrogen peroxide. The samples were incubated for 3 mins, and the reaction was stopped with 1 mL of chilled acetate buffer. The intensity of the blue chromogen of the reaction mixture (1 mL) that possessed a wavelength maximum at 655 nm was read. The number of units of MPO in the tissue homogenate was calculated from the plot of the standard MPO units vs. absorption. The MPO activity was recorded as number of units per mg of protein. Protein determination in the final homogenate was estimated by bicinchoninic acid (Pierce Chemical, Rockford, IL) protein method using total bovine serum albumin as a standard".

#### Serum DAO, Serum TNF- $\alpha$ , and Arterial Lactate ::: Materials and Methods:

The supernatants from the blood samples at T1, T2, and T3 were collected for measurements of DAO and TNF- $\alpha$ . Serum DAO activity and TNF- $\alpha$  concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO; Wuhan, China) performed according to the manufacturer's instructions. Each sample was run in duplicate and assessed by an automated ELISA reader at 436/450 nm wavelength. Arterial blood samples collected at T1, T2, and T3 in all groups were analyzed for blood lactate concentration using a portable blood gas analyzer (i-STAT; Abbott, Abbott Park, IL, USA).

#### Statistical Analysis ::: Materials and Methods:

Data were presented as means  $\pm$  SDs ( $n = 10$  rabbits/group). Significance was evaluated using one-way analysis of variance (ANOVA) (SPSS 21.0; SPSS Inc., Chicago, IL, USA) followed by Turkey's test for comparisons among groups. Statistical significance was set at  $P < 0.05$ . Correlations between different variables were assessed by Pearson's or Kendall's coefficient.

#### Hemodynamic Changes ::: Results:

No animals died prior to sacrifice at T3. BP levels were not different at T1 ( $P > 0.05$ ). The procedure for prolonged shock-resuscitation is detailed in Figure 1. The BP in the control group was relatively stable, fluctuating from 108 mm Hg to 96 mm Hg. The mean arterial pressure (MAP) decreased over an approximate 30-min period to the target shock BP of 41 to 50 mm Hg in the medium BP group and 31 to 40 mm Hg in the low BP group. The MAP during the shock maintenance phase was  $42.8 \pm 4.7$  mm Hg in the medium BP group and  $35.8 \pm 5.6$  mm Hg in the low BP group. The shock maintenance phase was successfully prolonged to 180 min to delay the resuscitation after hemorrhagic shock. At T3, the MAP in the low BP group ( $41.7 \pm 5.1$  mm Hg) was significantly lower than the MAP in the medium BP group ( $70.0 \pm 4.8$  mm Hg,  $P < 0.01$ ).

#### Intestinal Morphology ::: Results:

All animals were sacrificed at T3. The results of histological examination and Chiu's scoring are shown in Figure 2. According to Chiu's evaluating system, a higher Chiu's score represents more severe intestinal damage. In the control group, the intestines were normal with a Chiu's score of  $0.40 \pm 0.52$ . In the medium BP group, the intestines manifested lesions with a Chiu's score of  $2.3 \pm 0.67$ . In the low BP group, the intestines exhibited more severe damage with a Chiu's score of  $3.5 \pm 0.53$ . The scores were significantly different between binary comparisons of the groups ( $P < 0.01$ ).

#### Claudin-1 Expression ::: Results:

As aberrant claudin-1 expression directly impairs mucosal barrier function, immunohistochemistry was used to detect claudin-1 expression in our prolonged hemorrhagic shock model (Fig. 3). While a normal fluorescence intensity was seen in cross-sections of the control group, claudin-1 expression at T3 in the low and medium BP groups showed significantly decreased fluorescence intensity ( $P<0.05$ ), and the fluorescence intensity in the low BP group was significantly lower than that of the medium BP group ( $P<0.05$ ).

#### Intestinal ICAM-1 Expression ::: Results:

To understand the effects of ICAM-1 on intestinal neutrophil infiltration, ICAM-1 levels were evaluated in our prolonged hemorrhagic shock model (Fig. 4). The minimum fluorescence intensity for ICAM-1 was found in the intestinal mucosa of the control group, while the fluorescence intensity of ICAM-1 staining was significantly increased in the medium and low BP groups ( $P<0.05$ ). Moreover, ICAM-1 fluorescence intensity was significantly higher in the low BP group as compared to the medium BP group ( $P<0.05$ ).

#### Intestinal MPO Activity ::: Results:

As intestinal MPO activity is considered an index of polymorph nuclear leukocyte (PMN) sequestration [34], [35], MPO activity was assessed in our prolonged hemorrhagic shock model (Fig. 5). Compared with the control group ( $0.348\pm0.035$  IU/g), significantly increased MPO activity was found in the intestines of the medium BP group ( $0.615\pm0.047$  IU/g,  $P<0.01$ ) and the low BP group ( $0.911\pm0.068$  IU/g,  $P<0.01$ ). The low BP presented a significantly higher intestinal MPO activity than the medium BP group ( $P<0.01$ ). At T3, statistical analysis showed a negative correlation between the MPO activity and the claudin-1 expression ( $R = -0.937$ ,  $P<0.001$ ).

#### Serum TNF- $\alpha$ ::: Results:

To assess systemic inflammation, serum TNF- $\alpha$  was measured in our prolonged hemorrhagic shock model (Fig. 6A). During the experiment, serum TNF- $\alpha$  in the control group showed no significant change ( $P>0.05$ ). At T1, serum TNF- $\alpha$  values were not significantly different between binary comparisons of the control group ( $0.152\pm0.013$  ng/ml), the medium BP group ( $0.152\pm0.014$  ng/ml), and the low BP group ( $0.153\pm0.016$  ng/ml) ( $P>0.05$ ). However, at T2 and T3, serum TNF- $\alpha$  levels were significantly different between binary comparisons of the control group, the medium BP group, and the low BP group (T2:  $0.164\pm0.009$  ng/ml,  $0.215\pm0.009$  ng/ml, and  $0.319\pm0.016$  ng/ml, respectively,  $P<0.01$ ; T3:  $0.164\pm0.009$  ng/ml,  $0.362\pm0.020$  ng/ml, and  $0.626\pm0.042$  ng/ml,  $P<0.01$ ). These results indicate that a more severe hemorrhagic shock induces higher serum TNF- $\alpha$  concentrations in the prolonged hemorrhagic shock model, which indicates more severe systemic inflammation. At T3, statistical analysis showed a negative correlation between serum TNF- $\alpha$  and shock BP ( $R = -0.833$ ,  $P<0.01$ ) and a negative correlation with tissue claudin-1 expression ( $R = -0.958$ ,  $P<0.01$ ).

#### Serum DAO ::: Results:

To assess whether serum DAO can indicate the severity of intestinal damage, serum DAO was detected in our prolonged hemorrhagic shock model (Fig. 6B). At T1, no significant differences in serum DAO levels were detected between the control group and either the medium BP or low BP group ( $0.107\pm0.010$  IU/l vs.  $0.109\pm0.011$  IU/l or  $0.111\pm0.014$  IU/l, respectively,  $P>0.05$ ). At T2, serum DAO levels in the medium BP and low BP groups were both significantly higher than that in the control group ( $0.153\pm0.020$  IU/l and  $0.252\pm0.026$  IU/l vs.  $0.107\pm0.008$  IU/l, respectively,  $P<0.01$ ). A similar trend occurred at T3 ( $0.337\pm0.037$  IU/l and  $0.670\pm0.085$  IU/l vs.  $0.110\pm0.009$  IU/l, respectively,  $P<0.01$ ). Binary comparisons also revealed that the serum DAO level in the medium BP group were lower than that in the low BP group ( $0.153\pm0.020$  IU/l vs.  $0.252\pm0.026$  IU/l at T2,  $P<0.01$ ;  $0.337\pm0.037$  IU/l vs.  $0.670\pm0.085$  IU/l,  $P<0.01$ ). At T3, statistical analysis of the serum DAO content showed a positive correlation with serum TNF- $\alpha$  ( $R = 0.970$ ,  $P<0.01$ ) and a positive correlation with Chiu's score ( $R = 0.601$ ,  $P<0.01$ ).

#### Arterial Lactate ::: Results:

To assess hypoxia and hypoperfusion, arterial lactate was measured in our prolonged hemorrhagic shock model (Fig. 6C). No significant differences in arterial blood lactate were observed between all three groups at T1. Compared with the control group, anticipated increases in lactate levels occurred in the medium BP and low BP groups at T2 ( $18.97\pm1.52$  M and  $14.25\pm0.80$  mM vs.  $3.23\pm0.49$  mM, respectively,  $P<0.01$ ) and at T3 ( $10.29\pm1.02$  mM and

13.58±1.27 mM vs. 3.73±0.40 mM, respectively,  $P<0.01$ ). At T2, the low BP group manifested significantly higher lactate levels compared with the medium BP group (18.97±1.52 mM vs. 14.25±0.80 mM,  $P<0.05$ ); a similar finding was observed at T3 (10.29±1.02 mM vs. 13.58±1.27 mM). After fluid resuscitation, hypoxia and hypoperfusion were alleviated. As a result, the blood lactate concentration at T3 was lower than that at T2 in the medium BP group ( $P<0.05$ ) and the low BP group ( $P<0.05$ ). In the medium and low BP groups, serum lactate displayed an “increase after shock and decrease after resuscitation” trend.

#### Limitations :: Discussion:

Although our experiments confirmed that serum DAO levels reflect the severity of intestinal injury and elevated serum DAO levels are associated with increased degrees of systemic inflammation in a rabbit model, serum DAO's role as a potential indicator of the severity of prolonged hemorrhagic shock requires further study in clinical trials.

#### Conclusions:

In this study, we constructed a prolonged hemorrhagic shock animal model demonstrating that a more pronounced shock BP induces increased levels of systemic inflammation and intestinal mucosal barrier damage, suggesting that systemic inflammation possesses an etiopathological relationship with intestinal injury. The results reveal that serum DAO can reflect the severity of intestinal damage in prolonged hemorrhagic shock. Moreover, statistical analyses reveals serum DAO shows potential in evaluating the severity of prolonged hemorrhagic shock.