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Original Article

Role of using two-route ulinastatin injection to alleviate intestinal injury in septic rats

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ABSTRACT

Purpose: Early application of protease inhibitors through the intestinal lumen could increase survival following experimental shock by blocking the pancreatic digestive enzymes. Hence, it was hypothesized that two-route injection (intraintestinal + intravenous) of ulinastatin (UTI), a broad-spectrum protease inhibitor, could better alleviate intestinal injury than single-route injection (either intravenous or intraintestinal).

Methods: A sepsis model induced by lipopolysaccharide on rats was established. The rats were randomly divided into five groups: sham, sepsis, UTI intravenous injection (Uiv), UTI intraintestinal injection (Uii), and UTI intraintestinal + intravenous injection (Uii + Uiv) groups. The mucosal barrier function, enzyme-blocking effect, levels of systemic inflammatory cytokines, and 5-day survival rate were compared among groups. The small intestinal villus height (VH), crypt depth (CD), and two components of mucosal barrier (E-cadherin and mucin-2) were measured to evaluate the mucosal barrier function. The levels of trypsin and neutrophil elastase (NE) in the intestine, serum, and vital organs were measured to determine the enzyme-blocking effect.

Results: Compared with the single-route injection group (Uiv or Uii), the two-route injection (Uii + Uiv) group displayed: (1) significantly higher levels of VH, VH/CD, E-cadherin, and mucin-2; (2) decreased trypsin and NE levels in intestine, plasma, and vital organs; (3) reduced systemic inflammatory cytokine levels; and (4) improved survival of septic rats.

Conclusion: Two-route UTI injection was superior to single-route injection in terms of alleviating intestinal injury, which might be explained by extensive blockade of proteases through different ways.

Introduction

Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to the infection, represents a major health care challenge with a high incidence and mortality.[1] The mechanisms underlying sepsis-induced organ dysfunction remain poorly understood. Recent evidence demonstrated that pancreatic enzymes in the intestinal lumen are critical in triggering the systemic inflammation and multiple-organ dysfunction when the enzymes auto-digest the intestine.[2,3] Blocking these enzymes in the intestine increases survival in experimental models of sepsis.[4] However, the auto-digestion process may occur in the early stage of intestinal ischemia.[5] It is difficult to judge the time frame when administration of protease inhibitors through the intestinal route is sufficiently effective, especially in the clinical situation.

Ulinastatin (UTI) is an acidic glycoprotein purified from human urine. Compared with other protease inhibitors, UTI has a broader inhibitory activity. It could not only inhibit pancreas-derived proteases such as trypsin, chymotrypsin and elastase, but also effectively inhibit neutrophil elastase (NE), which is released from activated neutrophil granules and considered one of the most destructive enzymes in sepsis.[6,7] A recent randomized controlled

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trial (RCT) showed that early intravenous administration of UTI could significantly reduce the mortality of patients with severe sepsis by reducing new organ dysfunctions.[8] A few studies also showed a beneficial effect of UTI directly administered through the intraintestinal route in sepsis and intestinal ischemia-reperfusion models.[9] However, no study has compared and identified the way of UTI administration.

Considering the aforementioned uncertainties and the central role of intestinal mucosal barrier in the pathogenesis of sepsis, the present study was conducted using a rat model of sepsis induced by lipopolysaccharide (LPS). The main purpose was to investigate whether two-route UTI administration (intraintestinal + intravenous) might be superior to single-route administration (intraintestinal or intravenous) in alleviating sepsis-induced intestinal injury. The second purpose was to compare the effect of two different single-route UTI administrations: intraintestinal vs intravenous.

Methods

Laboratory animals

Clean-grade healthy male Wistar rats (age: 15–20 weeks, weight: 350–450 g) were purchased from Jianyang Dashuo Animal Science and Technology Co., Ltd, China. The animals were allowed to acclimatize for 3 days before use. They were raised on a standard chow diet with free access to food and water and housed in a temperature-controlled (21 °C ± 1 °C) room maintained under filtered positive-pressure ventilation on a 12-h light/12-h dark cycle beginning at 06:00. Prior to surgery the rats were restricted to solid food for 8 h with water *ad libitum*. All procedures were performed according to the protocols approved by the Animal Welfare Committee of West China Hospital (approval No.: 2016014A; approval date: May 25, 2016).

Main reagents and drugs

The drugs and chemicals used in this study and their corresponding sources are as follows: rat tumor necrosis factor alpha (TNF-α), trypsin, and NE enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, UK), rat interleukin-6 (IL-6) ELISA kit (J&L, Shanghai, China), antibodies of E-cadherin (E-cad) and mucin-2 (Abcam, Cambridge, UK), trypsin antibody (Lifespan, WA, USA), LPS from *Escherichia coli* serotype 055:B5 (Sigma, MO, USA), and UTI (TechpoolBio-Pharma Co, Ltd, Guangzhou, China).

Experimental model of sepsis

The rats ($n = 30$) were randomly divided into five groups: sham-operated group (sham), sepsis group without UTI administration (SS), and three sepsis groups treated with intraintestinal UTI (Uii), intravenous UTI (Uiv), and intraintestinal + intravenous UTI (Uii + Uiv), respectively.

All the experimental rats were anesthetized with intraperitoneal injections of 3% pentobarbital sodium (50 mg/kg). Then, the percutaneous tracheostomy was performed to keep the respiratory tract open. The right femoral artery catheterization was performed to monitor the mean arterial pressure, and the intravenous line was inserted into the caudal vein. After the aforementioned operations, a 5-min intravenous injection of LPS (5 mg/kg) was used to create the sepsis model and the lactated Ringer's solution [2 mL/(kg · h)] was pumped through the caudal vein to maintain the circulation. After achieving circulation stability, a midline incision (2.5 cm) was made to expose the small intestine as the intraintestinal administration route. Further, 10 WU UTI was dissolved in 5 mL 0.9% saline for intravenous injection, while UTI for intraintestinal administration was dissolved in 0.9% saline for a total fluid volume of 3 mL/100 g as described by Chang et al.[10] For example, a total fluid volume of 10.5 mL mixed with 1.75 WU UTI was injected into the lumen of the small intestine of a rat of 350 g. After 1 h of sepsis occurrence, 5 WU/kg UTI was slowly injected through different routes according to the group allocation. The dose of UTI was based on a previous report.[11] The intraintestinal administration followed the description by Delano et al.[4] The solutions were injected through 3–4 points of the intestine over its entire length from the duodenum to the ileum using a 1-mL needle. The sham and SS groups were treated with lactated Ringer's solution or 0.9% saline without any other intervention. After enzyme blockade, the intestine was returned into the peritoneal cavity gently, and the incision was closed with absorbable sutures in the abdominal muscle and silk sutures in the skin. Further, 3% pentobarbital sodium (50 mg/kg) was injected repeatedly every 3 h. During the whole experimental process, the rats were placed on the thermal insulation blanket to maintain their body temperature at 37 °C. The flow chart for experimental processes is shown in Fig. 1.

Sample collection

After 6 h of sepsis, 3 mL blood was collected through the femoral artery and centrifuged (3000 rpm, 15 min, 4 °C). The supernatant was frozen (−80 °C) for further analysis. The heart, lung, and partial jejunum were collected immediately after euthanasia (120 mg/kg sodium pentobarbital iv) and snap frozen for ELISA and Western blotting. The remaining jejunum tissues were excised and fixed (4% paraformaldehyde) for tissue histology. The lumen of the jejunum, as well as heart and lung sections, was rinsed with saline before freezing or fixation.

Measurement of intestinal mucosal barrier functions

Observation of villus height (VH) and crypt depth (CD) of the intestinal villi was conducted under a light microscope. The jejunum tissues were fixed, embedded, sectioned, and stained with hematoxylin/eosin for tissue histology. Images were processed using Motic Images Advanced 3.2 software, followed by measurements of the VH (measured from the tip to the crypt–villus junction) and CD (measured from the crypt–villus junction to the base). At least four measurements of VH or CD per explant were made.

Expression of E-cad andmucin-2 in the jejunum

Western blotting was used to confirm the level of E-cad and mucin-2. The jejunum tissues were lysed in RIPA buffer (Beyotime, Shanghai, China). The protein concentration was quantified using a Bicinchoninic Acid Protein Quantitative Kit (Keygen Biotech, Nanjing, China). An equal amount of protein (60 μg) was separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (Hybond, CA, USA), which were blocked with 5% nonfat milk for 2 h, incubated overnight at 4 °C with primary antibodies against E-cad (1:200 dilution), mucin-2 (1:2000 dilution), and β-actin (1:5000 dilution), followed by incubation with secondary antibody (1:5000 dilution) for 2 h, and detected by enhanced chemiluminescence.

Measurement of protease level in the jejunum

The jejunum level of trypsin was measured by Western blotting using the method described in the preceding section. The primary antibody against trypsin was diluted as 1:200.

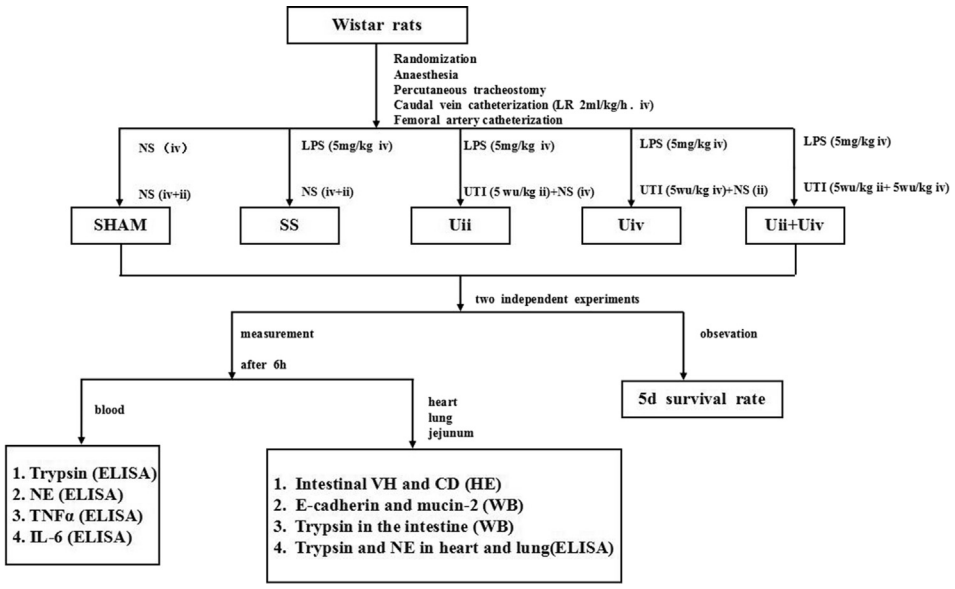

Fig. 1. A flow chart of experimental processes. LR: Lactated Ringer's solution; IV: Intravenous injection; II: Intraintestinal injection; NS: Normal saline; LPS: Lipopolysaccharide; UTI: Ulinastatin; NE: Neutrophil elastase; TNF-α: Tumor necrosis factor alpha; IL-6: Interleukin-6; ELISA: Enzyme-linked immunosorbent assay; WB: Western blot.

Measurement of cardiopulmonary and serum indexes

ELISA was conducted to measure the levels of TNF-α, IL-6, NE, and trypsin in serum, as well as the levels of NE and trypsin in cardiopulmonary tissues. The heart and lung were homogenized (0.1 g tissue/mL) in phosphate-buffered saline. Homogenates were centrifuged (1.4×10^4 g, 4 °C, 30 min), and the supernatants were collected and aliquoted for ELISA. The inspection was performed according to the kit instructions.

Effect of UTI on the survival rate

The rats were randomized into the sham group ($n = 10$), SS group ($n = 20$), Uii group ($n = 20$), Uiv group ($n = 20$), and Uii + Uiv group ($n = 20$). After 1 h of sepsis, 5 WU/kg UTI was slowly injected in the Uiv, Uii, and Uii + Uiv groups, while the sham and SS groups were given isometrical saline intraintestinally. The rats were all treated with fluid resuscitation by a subcutaneous injection of 50 mL/kg normal saline after the sepsis protocol. Then, they were kept under observation until anesthesia awareness and subsequently transferred into the vivarium. Survival was monitored every 2, 4, 6 and 8 h on the first, second, third, fourth day respectively, and every 24 h on the fifth day. The surviving rats were euthanized with sodium pentobarbital on postoperative day 5.

Statistical methods

Continuous variables were described as mean ± standard deviation (SD). One-way or two-way ANOVA followed by Bonferonni test were used for multiple comparisons and unpaired *t*-test or Wilcoxon test Comparisons were performed between independent groups. Multiple comparisons of survival among groups were performed using the Kaplan-Meier method. Statistical analysis was conducted using software SPSS 20.0 for Windows. Two-tailed tests of significance were employed, and significance was assumed at $p < 0.05$.

Results

Intestinal morphology and barrier function

The small intestinal VH, CD, and two components of mucosal barrier E-cad andmucin-2 were measured to assess the intestinal barrier function. E-cad is a transmembrane glycoprotein that connects epithelial cells together at adherent junctions.[12] The mucous layer, located on the outermost surface of the intestinal mucosal barrier, consists of various mucins (mucin-2).[13] Under the optical microscope (Fig. 2A), normal villi with intact epithelial cells were observed in the sham group. The completeness and regularity of jejunum villus were seriously damaged in the SS group. As presented in Figs. 2B and 2C, the situation improved after giving UTI in either the Uiv or the Uii group, which could also be explained by the higher levels of VH and VH/CD compared with the SS group. The most impressive finding was that compared with the Uiv or Uii group, the levels of VH and VH/CD were even higher in the Uii + Uiv group, which was similar to that in the sham group.

In addition, the expression of E-cad and mucin-2 presented a similar trend. Among all the groups, both E-cad and mucin-2 in the SS group decreased to the lowest level. After administering UTI, by either the intraintestinal or the intravenous route, E-cad and mucin-2 significantly increased compared with those in the SS group. No difference was observed between the Uii and Uiv groups. Furthermore, on comparing the Uii + Uiv group with either the Uii or Uiv group, E-cad and mucin-2 levels were much higher in the Uii + Uiv group, which recovered to the level in the sham group (Figs. 2D–2G).

Enzyme-blocking effect in the jejunum of the small intestine

The digestive enzymes were blocked via the lumen of the intestine to attenuate the enzyme access to the intestine and prevent the auto-digestive process. As shown in Fig. 3A, a significantly elevated level of trypsin was found in the SS group compared with the sham group in the intestinal wall. In all the treated sepsis

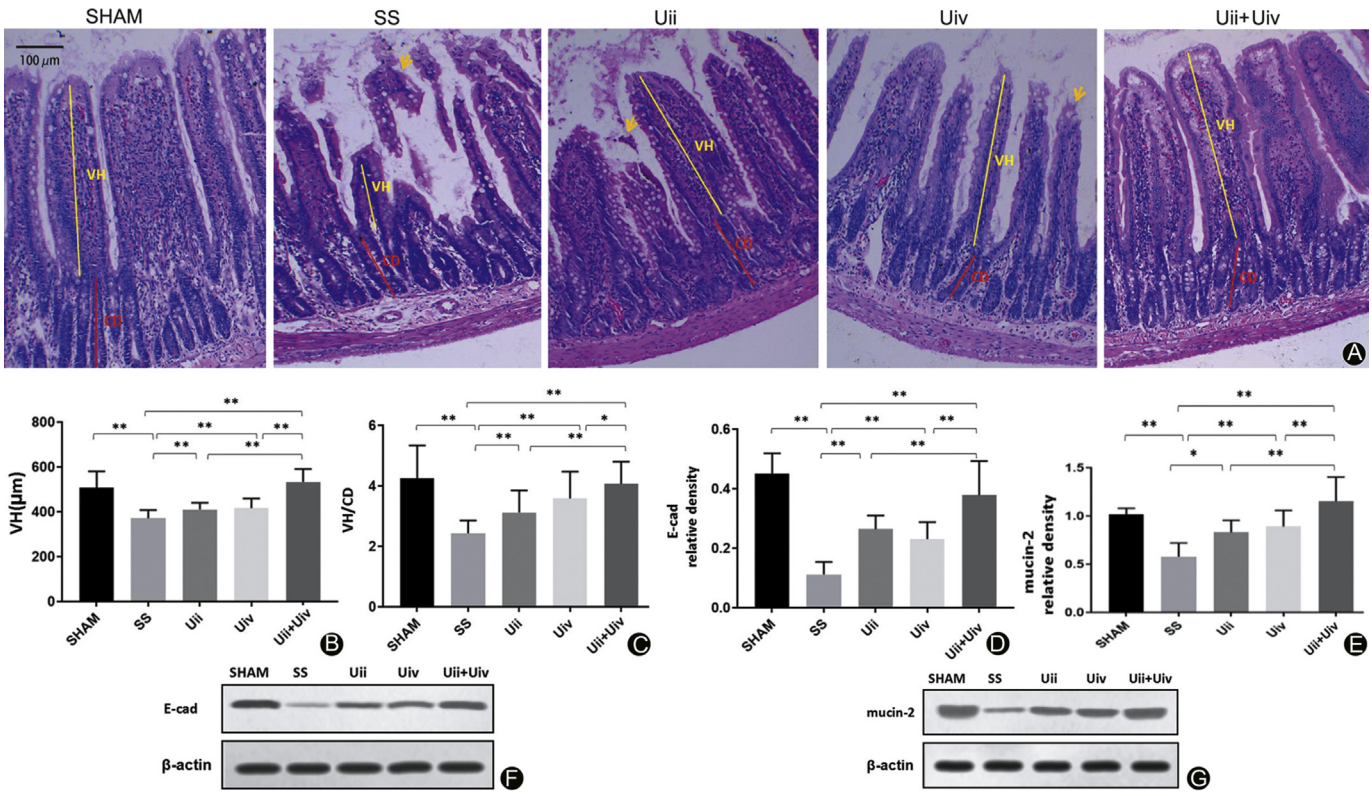

Fig. 2. Ulinastatin (UTI) alleviates intestinal injury in septic rats. A: Presentative micrographs of jejunal frozen sections stained with hematoxylin and eosin (original magnification ×100). Arrows show degeneration of villi. B, C: Villus height (VH) and VH/crypt depth (CD) measured using Motic Images Advanced 3.2. D, E: The densitometry quantifications for western blot results in Figs. F and G were performed via normalizing the band density of the indicated proteins to the loading control protein, respectively. F, G: Representative western blot analysis of E-cadherin (E-cad) and mucin-2 in jejunum homogenates after removing luminal contents. β-actin was used as an endogenous control. $n = 6$ rats for each group.

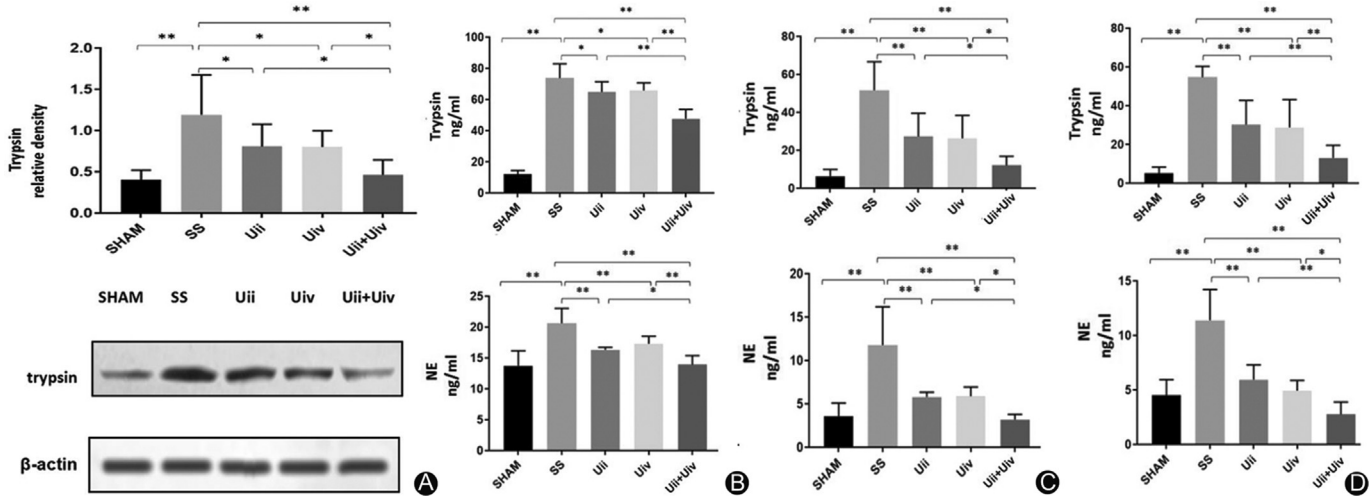

Fig. 3. Enzyme-blocking effects of Ulinastatin (UTI) in septic rats. A: The protein levels of trypsin in the jejunum tissues were determined by western blotting. β-actin was used as an endogenous control. Levels of trypsin and neutrophil elastase (NE) in (B) serum, (C) heart, and (D) lung, measured using enzyme-linked immunosorbent assay (ELISA). $n = 6$ rats for each group. Data are shown as averages ±SD. The comparisons between the groups were made with one-way ANOVA. $^{**}p < 0.01$, $^{*}p < 0.05$. SHAM = sham-operated group; SS = sepsis group without UTI administration; Uii = sepsis group treated with intraintestinal UTI; Uiv = sepsis group treated with intravenous UTI; Uii + Uiv = sepsis group treated with intraintestinal + intravenous UTI.

models that have been tested, the average levels of trypsin significantly decreased in the intestinal wall ($p < 0.05$, treated sepsis versus SS group). The two-route UTI injection (Uii + Uiv) was more effective than either Uii or Uiv injection alone.

Enzyme-inhibitory effects in the serum and vital organs

The trypsin and NE levels in the serum and vital organs were determined to explore the enzyme-inhibitory effects of UTI

(Figs. 3B–3D). The serum levels of trypsin and NE significantly increased in the SS group than in the sham group. However, these changes in septic rats were reduced by both intraintestinal and intravenous administration of UTI, and the effects of two-route injection (Uii + Uiv) were much better than those of Uii or Uiv.

Similar to the aforementioned results, the cardiopulmonary levels of trypsin and NE significantly increased in the SS group than those in the sham group. Intraintestinal + intravenous injection of UTI greatly inhibited these proteases and was much more effective than either Uii or Uiv injection alone.

Anti-inflammatory effects

Excessive inflammatory reaction is vital in the pathogenesis of sepsis. The effects of different administration methods of UTI on anti-inflammatory reactions were evaluated by testing the serum levels of TNF-α and IL-6, which are associated with systemic inflammation. As shown in Fig. 4, LPS injection dramatically increased inflammatory cytokine levels in the serum of the SS group. After administering UTI, by either intraintestinal or intravenous route, the serum levels of TNF-α and IL-6 significantly decreased compared with that in the SS group. Consistent with the aforementioned results, the Uii + Uiv group showed the strongest anti-inflammatory effects among all the UTI-treated sepsis groups.

Survival of septic rats

The effects of UTI on 5-day survival of septic rats were observed (Fig. 5). The results showed that septic rats treated with UTI had a significantly longer survival time compared with those in the SS group ($p < 0.05$). Furthermore, on comparing the Uii + Uiv group with either the Uii or Uiv group, the survival time was much longer in the Uii + Uiv group.

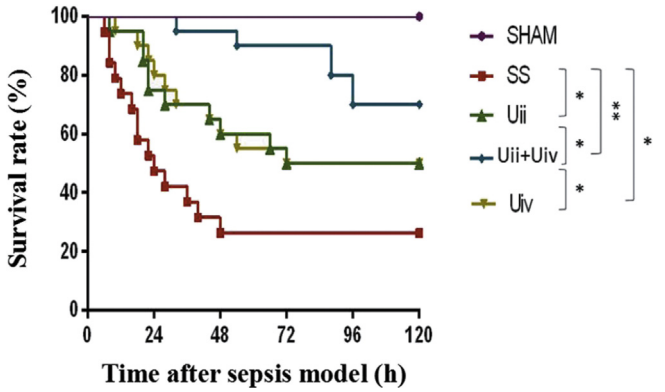

Fig. 5. Effects of administration of ulinastatin (UTI) on the survival of rats with sepsis. Septic rats were intraintestinally (ii), intravenously (iv) or intraintestinally + intravenously (ii + iv) given 50,000-units/kg UTI or isometrical normal saline at 1 h after sepsis. The whole period of follow-up is compared with a Kaplan-Meier analysis and Breslow test. $^{**}p < 0.01$, $^{*}p < 0.05$. SHAM = sham-operated group ($n = 10$); SS = sepsis group without UTI administration ($n = 20$); Uii = sepsis group treated with intraintestinal UTI ($n = 20$); Uiv = sepsis group treated with intravenous UTI ($n = 20$); Uii + Uiv = sepsis group treated with intraintestinal + intravenous UTI ($n = 20$).

Discussion

Based on the LPS-induced sepsis model, this novel study compared three different methods (intraintestinal, intravenous, or intraintestinal + intravenous injection) of UTI administration in the early stage of sepsis. It showed that the two-route (intraintestinal + intravenous) administration of UTI significantly improved the intestinal function by decreasing enzyme insult.

Theoretically, protease-induced inflammation is one of the important factors contribute to the high mortality of sepsis.[4,14] A previous study proposed that pancreatic enzymes could escape into the injured intestine, entered the bloodstream, and caused a cascade of inflammatory reactions, which had a central role in sepsis progression.[2,15] It is possible that blockade of digestive enzymes via the lumen of the intestine may alleviate the deleterious effect. Further, DeLano et al.[4] confirmed using the sepsis shock model that intraintestinal administration of proteinase inhibitors 6-amidino-2-naphthyl *p*-guanidinobenzoatedimethanesulfate or tranexamic acid could inhibit the activities of digestive enzymes, ameliorate the expression of inflammatory mediators, and increase the survival rate. In addition, NE, a serine protease that propagates persistent neutrophilic inflammation by attacking host proteins of neutrophils or accelerating pro-inflammatory cytokine production, may also participate in the development of sepsis.[16,17] Such proteolysis may change the protein pattern of an inflammatory focus depending on the number of neutrophils involved and the duration of inflammation.[14] Of note, Suda et al.[18] found that a specific NE inhibitor improved the survival of animals with sepsis. Intestinal tissue and other vital organs are susceptible to the direct and indirect effects of both trypsin and NE. Hence, it is reasonable to hypothesize that two-route UTI injection (intraintestinal + intravenous) would be beneficial. This study found that the two-route administration of UTI was able to reduce intestinal injury, enzyme insult, and inflammatory response.

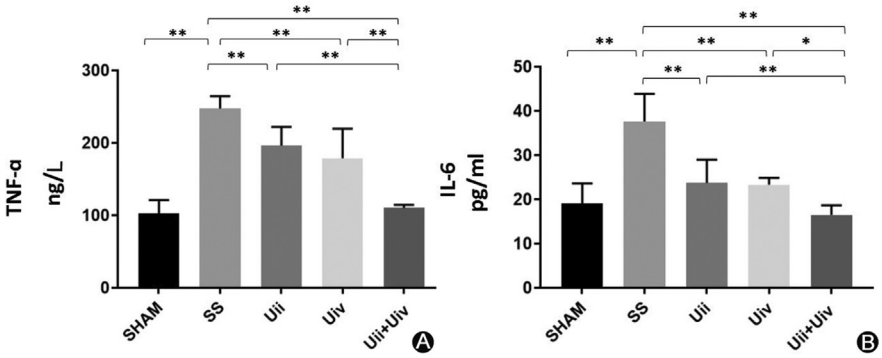

Fig. 4. Anti-inflammatory effects of Ulinastatin (UTI) in septic rats. Levels of (A) tumor necrosis factor alpha (TNF-α) and (B) interleukin-6 (IL-6) in serum measured using enzyme-linked immunosorbent assay (ELISA). $n = 6$ rats for each group. Data are shown as averages ±SD. The comparisons between the groups were made with one-way ANOVA. $^{**}p < 0.01$, $^{*}p < 0.05$. SHAM = sham-operated group; SS = sepsis group without UTI administration; Uii = sepsis group treated with intraintestinal UTI; Uiv = sepsis group treated with intravenous UTI; Uii + Uiv = sepsis group treated with intraintestinal + intravenous UTI.

Methodologically, this study provided evidence to support the role of two-route UTI injection in treating sepsis. It showed that the two-route administration of UTI minimized damage to the mucin mucosal layer and E-cadherin junctions in the intestine, thereby preserving the morphology of the villi. To exclude the influence of hemodynamic changes on intestine barrier, we pumped lactated Ringer's solution [2 mL/(kg·h)] to maintain the circulation after injection of LPS. The MAPs were similar among all the sepsis groups in the first 6 h. In addition, the intestinal, serum, and cardiopulmonary levels of trypsin, NE, TNF-α, and IL-6, and 5-day survival were also observed, which displayed a better effect in the two-route UTI injection and hence made the study more convincing.

Clinically, UTI is used mainly to treat pancreatitis, peripheral circulatory failure, and severe sepsis through the intravenous route in Asia.[19,20] RCTs of UTI as a therapeutic, both as a single drug[9,21] and in combination with the immunomodulatory agent thymosin-α1,[22,23] showed beneficial effects such as a significant improvement in inflammatory markers and, to a lesser extent, in organ dysfunction. The present study provided not only evidence for the rational use of UTI in the future, but also a new idea for the use of other anti-protease or anti-inflammatory drugs.

The present study had some limitations. Firstly, in the initial design of the experiment, we focused on whether the proteases could permeate through the mucus of the intestine, so we measured the protease levels in the jejunum and remote organs. However, it could not be neglected that the protease bioactivities in the intestine lumen fluid is also very important. In the future work, we should detect the protease levels in intestinal lumen fluid to determine the optimal dosage of UTI through intestine route. Secondly, we gave 5 WU/kg of UTI through IV or II route separately in the two-route injection group. However, we don't know exactly whether the UTI could be absorbed from the intestine and whether the single-route injection could also get the same effect if we double the dosage. These questions also should be clarified in the further research. Thirdly, UTI was administered directly into the lumen of the intestine in an invasive manner to ensure the uniformity of drug delivery site. Hence, optimal delivery methods need to be developed. In this regard, it is noteworthy that a patient with severe septic shock was successfully treated with continuous enteral protease inhibitor via a nasogastric tube.[24] Hence, noninvasive drug delivery can be performed in the future with the help of imaging techniques to determine the location of administration.

In conclusion, this study provided evidence to support that the two-route UTI injection was superior to the single-route injection in terms of alleviating intestinal injury, which might be explained by extensive blockade of proteases through different ways. Meanwhile, this study suggested that the two-route UTI injection was a promising anti-protease and anti-inflammatory way that could be useful as a therapeutic method in sepsis. However, this approach needs to be further evaluated in future studies. The key requirement is to reduce the activity of proteases once they have escaped from the lumen of the intestine and minimize their leakage.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cjtee.2018.05.002.

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