Urinary trypsin inhibitor treatment ameliorates acute lung and liver injury resulting from sepsis in a rat model

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ABSTRACT

الأهداف: من أجل تقييم الأثر الوقائي للحاصر التريبسين البولي (UTI) على إصابة الكبد والرئة الحادة في نموذج الجرذان المحرض بواسطة الإنتان مع الالتهاب فيما دون البطن.

الطريقة: أجريت هذه الدراسة بجامعة ووهان في مايو 2007م. تم عمل نماذج الانتان بواسطة ربط الأعوري وإجراء الثقب (CLP) في جرذان من نوع سبارجيو– داولي. تم تقسيم 40 جرذ بشكل عشوائي الى مجموعة مصابة ومجموعة (CLP) ومجموعة (UTI I/CLP) (20u/g) ومجموعة (UTI/CLP) (50u/g) بعدد 10 جرذان في كل مجموعة. تم التضحية بالجرذان جميعهم عند الساعة 12 بعد إجراء (CLP). تم قياس الضغط الشرياني الرئيسي (MAP) ومعدل نبض القلب (HR) ومستوى وزن الرئة الرطب الى الجاف وتم جمع عينات الدم من أجل معايرة عامل نكرزة الورم–a (TNF–a) وانترليوكين 10– (IL–10) وألانين أمينوترانسفيرس (ALT) وأسبريتيت أمينوترانسفيرس (AST) وحمض اللاكتيك. تم فحص ديسموتاس فوق الأكسدة (SOD) ومالوندياليديهيد (MDA) وتعبير نتريك أوكسيد سينثيس المحرض (iNOS) في أنسجة الرئة والكبد.

النتائج: بمقارنة مجموعة (CLP) ومجموعة (MAP) ومجموعة (HR) في الجرذان التي تلقت 50 ميكروجرام كانت مستقرة نسبياً ($p>0.01$). كانت مستويات الرفع المؤشرة لمعدل الرطوبة/الجفاف أقل بعد تلقي 50 ميكروجرام من (UTI) ($p>0.01$) (UTI). المعالجة بواسطة 50u/g من (UTI) الوقائي ارتفع في مستويات (MDA) و(TNF–α) (AST) وحمض اللاكتيك وتعبير (iNOS) ونشاط آي 10-IL و(SOD) المرتفع ($p>0.01$).

خاتمة: لدى الحاصر التريبسين البولي (UTI) تأثير وقائي ضد الإنتان. فآلية العمل لديه تتعلق في تثبيط إنتاج عامل الإلتهاب والدهون فوق الأكسدة وتعبير (mRNA) (iNOS).

Objective: To evaluate the protective effect of urinary trypsin inhibitor (UTI) on acute lung and liver injury in rat model induced by sepsis with infra-abdominal infection.

Methods: This study was performed in the University of Wuhan, Wuhan, China in May 2007. Sepsis models were made by cecal ligation and puncture (CLP) in Sprague-Dawley rats. Forty rats were randomly divided into sham, CLP, CLP/UTI I (20u/g) and CLP/UTI II (50u/g) groups, with 10 rats in each. All of them were sacrificed 12 hours after CLP. The mean arterial pressure (MAP), heart rate (HR), the wet-to-dry lung weight ratio (W/D) was measured and venous blood was collected for assaying tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactic acid. Superoxide dismutase (SOD), malondialdehyde (MDA) and expression of inducible nitric oxide synthase (iNOS) mRNA in lung and hepatic tissues were examined.

Results: Compared with the CLP group, MAP and HR in 50u/g UTI treated rats was stable ($p<0.01$). Marked elevation levels of W/D ratio were lowered after administration of 50u/g UTI ($p<0.01$). Treatment with 50u/g UTI prevented marked elevation in MDA, ALT, AST, TNF-α, lactic acid levels, expression of iNOS mRNA, and elevated IL-10 and SOD activity ($p<0.01$).

Conclusion: Urinary trypsin inhibitor has a protective effect against sepsis. Its action mechanisms are probably involved in the inhibition of inflammatory factor production and suppression of lipid peroxidation and iNOS mRNA expression.

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Urinary trypsin inhibitor (UTI), a protease inhibitor, is an acidic glycoprotein that is discovered in human blood and urine.[1] Urinary trypsin inhibitor has been extensively administered for patients with shock and pancreatitis. It suppresses proteases such as trypsin, chymotrypsin, and elastase, as well as stabilizes lysosomal membranes, and thereby inhibits the release of lysosomal enzymes.[2] Urinary trypsin inhibitor has anti-inflammatory characteristics and inhibits the enhanced production of proinflammatory molecules such as interleukin-8 (IL-8),[3] tumor necrosis factor-a (TNF-α)[4] induced by lipopolysaccharide (LPS). In addition, UTI ameliorates inflammatory models such as hemorrhagic shock,[5] and glomerulonephritis[6] in vivo. Septic shock is a sophisticated pathophysiologic procedure characterized by hypotension, metabolic acidosis, systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), and even death.[7] Sepsis and septic shock are the cardinal agents of morbidity and mortality in critically ill patients.[8] During the onset of sepsis, inflammatory cytokines, and oxyradicals have been implicated in the sophisticated host-pathogen interaction underlying organ damage and multi-system organ failure, which are signs of sepsis and common causes of death. In addition to this systemic inflammatory process, sepsis and septic shock cause a profound increase in inducible nitric oxide synthase (iNOS) messenger ribonucleic acid (mRNA) levels, leading to a great increase in local nitric oxide (NO) levels.[9,10] In this study, we explored the effectiveness of UTI in acute lung and liver injury induced by septic shock resulting from cecal ligation and puncture (CLP). In the present experiment, TNF-α, IL-10, lactic acid in serum, superoxide dismutase (SOD), malondialdehyde (MDA), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and expressions of iNOS mRNA in lung and hepatic tissues in septic rats with UTI were examined.

Methods. Urinary trypsin inhibitor was provided by Techpool Bio-Pharma Corporation (Guangdong, China), SOD and MDA kits were purchased from Jiancheng Biologic Project Company (Nanjing, China), enzyme-linked immunosorbent assay (ELISA) kit was available from Jingmei Corporation (Shenzhen, China), Healthy Spargue-Dawfey (SD) rats (male, 230±20 g) were provided by the Department of Laboratory Animal Center of Wuhan University, Wuhan, China.

Groups and experimental protocols. Experiment was performed in University of Wuhan, Wuhan, China in May 2007. The following investigations were carried out under ethical approval by the Animal Care Committee of Wuhan University. Animal experiments were performed in accordance with the national legislation. Forty rats were randomly divided into 4 groups: sham controls, CLP alone, and CLP treated with 20u/g UTI (CLP/UTI I) or 50u/g UTI (CLP/UTI II) (n = 10 rats each). In UTI groups, rats were intraperitoneally injected with different doses of UTI (20u/g or 50u/g) immediately after performing CLP and 6 hours after performing CLP (20u/g or 50u/g). Sham controls and CLP-alone rats received the same volume of saline. For CLP, rats were anesthetized, the cecum was ligated with a 3-0 silk ligature, punctured with a 9-gauge needle, and a small amount of cecal contents was extruded from the perforation sites. Incisions were closed in layers. Sham control rats had laparotomy without CLP.

Measurement of mean arterial pressure (MAP) and heart rate (HR). After a cervical incision was made on rats anesthetized with chloral hydrate (500mg/kg, intraperitoneally), a stretched PE10 polyethylene catheter filled with heparinized saline was inserted into the right common carotid artery. A ligature was then tied around the artery, and the catheter was tunneled subcutaneously to exit at the back of the neck. The arterial cannula was connected to a pressure transducer (Mindray PM-9000, China). Systolic and diastolic arterial blood pressure as well as MAP was recorded online. Measurement of HR was triggered from changes in arterial pressure. Time points "0" were aligned at the point when right common carotid artery catheterization and MAP, HR, values were measured.

Sample preparation. Blood plasma, lung, and liver tissue samples were collected 12 hours after surgery. All blood samples were centrifuged immediately at 2,500 rpm for 15 minutes at 4°C. Plasma supernatant was collected and stored at -20°C. Tissue samples were frozen and stored at -70°C for index assessment.

Measurement of MDA and SOD. Whole lung and liver were homogenized in 0.9% saline solution using a homogenizer. The homogenate was then centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant obtained was used for assays of MDA and SOD activities. Malondialdehyde content was determined by the thiobarbituric acid method, whereas SOD activity was evaluated according to the xanthine oxidase method. The absorbance was measured at 532 nm for MDA and 550 nm for MDA and SOD, with a spectrometer (Jiancheng Biologic Project Company, Nanjing, China). Each measurement was performed in duplicate. Malondialdehyde concentration was expressed as nanomoles per milligram of protein, SOD activity was expressed as units per milligram of protein.

Measurement of lung W/D ratio, ALT, AST, and lactic acid. Wet/dry weight ratios were measured as indexes of lung injury. Lung tissues were taken from the upper and lower lobes and weighed, and then evaporated in an oven kept at 80°C for 24 hours and weighed again. Lung W/D weight ratios were calculated

and expressed as relative values. Liver function was examined by measuring plasma levels of ALT and AST. The plasma concentrations of ALT, AST, and lactic acid were measured with the use of an OLYMPUS AU5400 automatic analyzer in accordance with the manufacturer's instructions. Alanine aminotransferase and AST values were expressed as units per liter, and lactic acid was expressed as millimoles per liter.

Measurements of TNF-α, IL-10, and polymerase chain reaction for iNOS mRNA. Plasma TNF-α, IL-10 were measured by ELISA method using commercially available kits (Jingmei Corporation, China) according to the manufacturer's instructions. In brief, the ELISA method (performed at room temperature) was as follows: Add 100uL per well of the diluted capture antibody. Seal the plate and incubate overnight. Aspirate each well and wash with wash buffer, repeating the process 2 times for a total of 3 washes. Add 100uL of rat plasma per well then cover with an adhesive strip and incubate for 2 hours. After washing the plate, the wells were incubated for 2 hours with 100uL of the detection antibody. Thereafter the plate was washed; add 100uL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. Sample concentrations were calculated using the proper standard calibration lines. Total cellular RNA was isolated from the lung and liver tissue of each rat using Trizol reagent following the protocol recommended by the manufacturer. Inducible nitric oxide synthase expression was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR).[11] β-actin was used as the gene transcript control. Primers used for iNOS (298bp) were 5'-AACGCTACACTTCCAACGCA-3' (upstream) and 5'-GGAGCGAGTTGTGGATTGTTC-3' (downstream). Primers used for β-actin (184bp) were 5'-CAATTCCATCATGAAGTGTGAC-3' (upstream) and 5'-CCACACAGAGTACTTGCGCTC -3' (downstream). The PCR conditions were initial denaturation at 94°C for 3 minutes, followed by 35 cycles at 94°C (denaturation) for one minute, 60°C (annealing) for 45 seconds and 72°C (primer extension) for one minute, and finally 72°C for 7minutes. After the RT-PCR reaction, the PCR products of iNOS and β-actin were analyzed by 2% agarose gel electrophoresis. Densitometric analysis for semi-quantitation of the PCR products was performed with an alpha imager 2000 analyzer. Expressions of the ratio of the iNOS/β-actin were calculated.

Statistical analysis. Data were presented as mean ± SD. Data processing were performed using SPSS version 12. The ANOVA and Student-Newman-Keuls q test were used for statistical analysis to compare measurement data among all groups. Differences were considered significant with probability values of less than 0.05.

Results. ***Mean arterial pressure and HR.*** Changes of MAP and HR in 4 groups of rats over the observation period are presented in **Figures 1 & 2**. Changes during the first 6 hours had no significant differences in MAP and HR. At 8, 10, and 12 hours, MAP was significantly decreased in CLP rats ($p<0.01$) versus sham controls, whereas MAP in 50u/g UTI treated rats was stable. Cecal ligation and puncture rats had significantly higher HR values than corresponding sham controls at 8, 10, and 12 hours after operation ($p<0.01$), whereas fluctuation in HR was stable following the administration of 50u/g UTI. The baseline MAP was 105±5 mmHg, and HR was 370±8 bpm.

Malondialdehyde content, SOD activity in lung and liver. The roles of UTI on MDA contents and SOD activities in lung and liver tissues were investigated, and these data are summarized in **Table 1**. Lung MDA levels in CLP rats were significantly higher than in sham controls ($p<0.01$). Significantly increased MDA levels were also observed in the liver of CLP group when compared with sham controls ($p<0.01$). There is no significant difference between the sham and 50u/g UTI group of the MDA contents in liver tissues ($p>0.05$). Malondialdehyde and SOD in 20u/g UTI and 50u/g UTI groups have significant difference ($p<0.01$). Superoxide dismutase activities were significantly lower in CLP rats compared with sham control rats ($p<0.01$). Treatment with 20u/g and 50u/g UTI prevented marked elevation in MDA levels and reduction in SOD activities ($p<0.01$).

Measurement of lung W/D ratio, ALT, AST, and lactic acid. Results of lung W/D weight ratios are presented in **Table 2**. The W/D ratio of the lung in CLP rats was markedly increased ($p<0.01$) versus

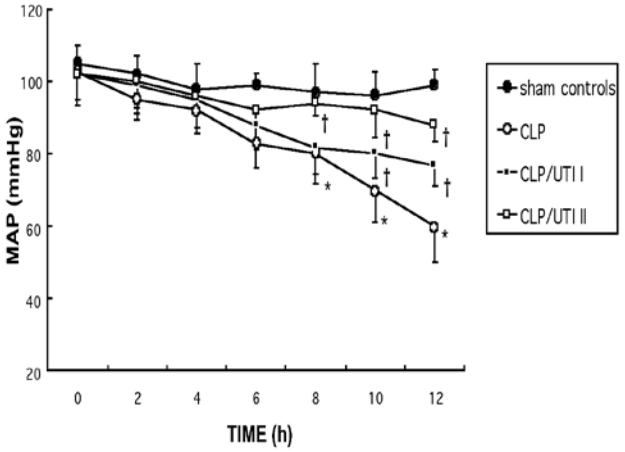

Figure 1 - Changes of mean arterial pressure in 4 groups of rats over the observation period. Compared with sham controls $^{*}p<0.01$, compared with cecal ligation and puncture (CLP) group $^{\dagger}p<0.01$.

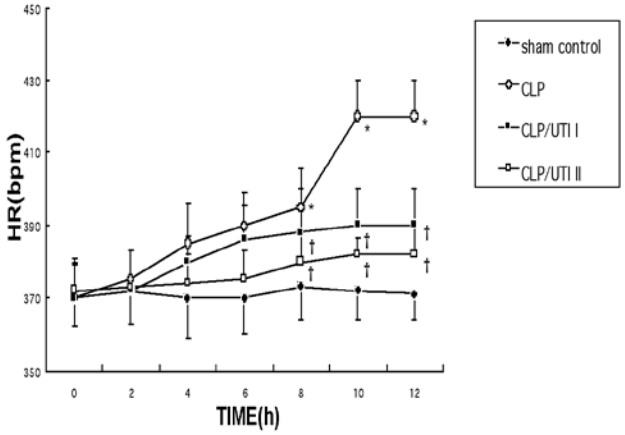

Figure 2 - Changes of heart rate in 4 groups of rats over the observation period. Compared with sham controls $^{*}p<0.01$, compared with cecal ligation and puncture (CLP) group $^{\dagger}p<0.01$.

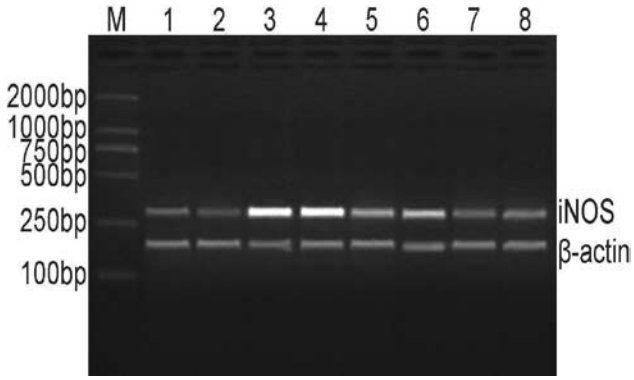

Figure 3 - The expression of inducible nitric oxide synthase mRNA. Illustration of M marker: 1 - sham controls lung, 2 - sham controls liver, 3 - cecal ligation and puncture (CLP) lung, 4 - CLP liver, 5 - CLP/UTI I lung, 6 - CLP/UTI I liver, 7 - CLP/UTI II lung, 8 - CLP/UTI II liver, bp - base pair.

Table 1 - Changes of MDA content and SOD activity.

Group	MDA (nmol/mg protein)		SOD (U/mg protein)	
	Lung	Liver	Lung	Liver
Sham controls	1.68±0.05	0.81±0.03	147.0±1.41	394.8±20.2
CLP	2.01±0.04*	1.45±0.03*	121.8±2.86*	266.6±23.8*
CLP/UTI I	1.85±0.03†	1.21±0.16†	135.2±2.66†	327.7±13.1†
CLP/UTI II	1.74±0.03†‡	0.85±0.01†‡	140.0±1.33†‡	360.3±10.2†‡

MDA - malondialdehyde, SOD - superoxide dismutase, CLP - cecal ligation and puncture, UTI - urinary trypsin inhibitor. Each value is the mean ± SD, $^{*}p<0.01$ compared with sham controls, $^{\dagger}p<0.01$ compared with CLP group, $^{\ddagger}p<0.01$ compared with UTI I group

Table 2 - Changes of lung W/D ratio and parameters of liver enzyme, lactic acid (Lac).

Group	W/D	ALT (U/L)	AST (U/L)	Lac (mmol/L)
Sham controls	4.07±0.16	81.5±17.8	70.0±6.0	0.96±0.13
CLP	5.38±0.25*	143.2±40.1*	138.2±10.0*	3.09±0.22*
CLP/UTI I	4.91±0.12†	110.5±20.3†	100.0±11.8†	2.11±0.19†
CLP/UTI II	4.56±0.14†‡	92.7±14.6†‡	81.9±6.2†‡	1.45±0.3†‡

W/D - wet/dry weight ratio, ALT - alanine aminotransferase, AST - aspartate aminotransferase, CLP - cecal ligation and puncture, UTI - urinary trypsin inhibitor. Each value is the mean ± SD, $^{*}p<0.01$ compared with sham controls, $^{\dagger}p<0.01$ compared with CLP group, $^{\ddagger}p<0.01$ compared with UTI I group

Table 3 - Changes of TNF-α, IL-10 concentration, and iNOS mRNA.

Group	TNF-α (pg/ml)	IL-10 (pg/ml)	iNOS mRNA (iNOS/β-actin)	
			Lung	Liver
Sham controls	4.67±0.4	98.6±6.15	0.78±0.06	0.71±0.06
CLP	10.25±0.5*	75.5±4.14*	2.07±0.11*	2.19±0.22*
CLP/UTI I	8.7±0.9†	83.7±2.67†	1.56±0.11†	1.61±0.12†
CLP/UTI II	6.83±0.9†‡	95.4±5.29†‡	1.19±0.11†‡	1.20±0.11†‡

TNF-α - tumor necrosis factor-α, IL-10 - interleukin-10, iNOS - inducible nitric oxide synthase, CLP - cecal ligation and puncture, UTI - urinary trypsin inhibitor. Each value is the mean ± SD, $^{*}p<0.01$ compared with sham controls, $^{\dagger}p<0.01$ compared with CLP group, $^{\ddagger}p<0.01$ compared with UTI I group

sham control rats, indicating that CLP induced the lung edema. Marked elevation levels of W/D ratio were lowered after administration of 20u/g and 50u/g UTI (p<0.01). Results of serum ALT, AST, and lactic acid levels are presented in **Table 2**. A marked increase in the activity of ALT and AST was observed in CLP rats when compared with sham controls (p<0.01). Rats treated with 20u/g UTI and 50u/g UTI showed a significant (p<0.01) decrease in ALT and AST levels. Serum ALT and AST in 20u/g UTI and 50u/g UTI groups have significant difference (p<0.01). All indices showed that CLP rats had serious tissue injury, whereas UTI treatment can improve tissue status and maintain cellular integrity. In the CLP group, serum lactic acid was significantly higher than in sham controls (p<0.01), whereas after treatment with UTI, serum lactic acid was remarkably lower than in CLP rats (p<0.01).

The concentration of TNF-α, IL-10, and expression of iNOS mRNA. Results of the concentration of TNF-α and IL-10 are presented in **Table 3**. CLP caused a marked increase of TNF-α concentration compared with sham control (p<0.01). The application of UTI reduces this value significantly. In rats treated with 20u/g and 50u/g UTI, TNF-α plasma concentration was significantly lower than in the rats after CLP (p<0.01). Interleukin-10 plasma concentration was decreased after CLP, and the difference was significantly compared with sham controls (p<0.01). Compared with CLP group, UTI administration can elevate the concentration of IL-10 (p<0.01). There is no significant difference between the sham and 50u/g UTI group (p>0.05). The results of iNOS mRNA expression are presented in **Table 3** and **Figure 3**. Inducible nitric oxide synthase mRNA expression in tissues was increased after CLP, and the difference was significantly compared with sham controls (p<0.01). Compared with CLP group, UTI administration can inhibit iNOS mRNA expression in lung and liver tissues (p<0.01).

Discussion. The murine CLP model has been shown to induce a polymicrobial sepsis. With CLP, abdominal cavity is contaminated by intestinal contents, resulting in diffuse peritonitis. Septic shock results in a blood volume deficit secondary to fluid loss from capillary leak. The poor perfusion contributes significantly to the pathophysiology of sepsis.[12] In our study, CLP produced progressive hypotension, and obvious increase in plasma cytokine concentrations. Meanwhile, the CLP model causes acute hepatic and lung injury in rat, which manifests the significant elevation of W/D ratio of the lung, ALT, and AST. Considering the possible roles of proteases in inflammatory diseases, UTI would be expected to have a beneficial effect on inflammatory disorders. In Molor-Erdene[13] experiment, UTI inhibited lipopolysaccharide (LPS)-induced increases in lung tissue levels of TNF-mRNA, and TNF-α in rats. Urinary trypsin inhibitor inhibited LPS-induced hypotension by inhibiting pulmonary induction of iNOS. Our studies showed that UTI might have a potential protective effect against sepsis in many ways. Urinary trypsin inhibitor significantly attenuated tissue damage and elevation of plasma lactate, illustrating that UTI can improve metabolic acidosis and prevent cell and organ damage during septic progression. The mechanism of UTI protection on sepsis may be related to the following factors: Urinary trypsin inhibitor can counteract lipid peroxidation and enhance the ability of SOD clearing oxyradical. In sepsis, bacteria and endotoxin directly act on phagocyte and result in lipid peroxide formation and membrane damage in tissues of experimental animals, causing tissue injury.[14] The results of our study showing elevated MDA content and reduced SOD activity in CLP rats' tissues during sepsis. In the 50u/g UTI group, MDA levels were significantly lower than those of CLP group (p<0.01); meanwhile, SOD activities were obviously higher than those of CLP group (p<0.01). The improved result was likely due to antioxidant activity since UTI prevents stimulation by endotoxin of free radical production. Urinary trypsin inhibitor can significantly inhibit production of TNF-α. Tumor necrosis factor-α is thought to be the core of the cytokine network, which is a critical early mediator in the origin of a systemic inflammatory response during a septic detriment. The plasma concentration of TNF-α is correlated both with the severity of sepsis and with the extent of subsequent MODS.[15,16] Our studies found that plasma TNF-α was markedly increased after CLP, whereas after treatment with UTI, plasma TNF-α was significantly lower in CLP rats (p<0.01), demonstrating that UTI can inhibit TNF-α generation.

Cytokine IL-10 has a suppressive effect on the inflammation immunologic response, protection effect on the body and has conduced to control systemic inflammation. Our studies found that IL-10 plasma concentration was decreased after CLP, and the difference was significantly compared with sham controls (p<0.01). Compared with CLP group, UTI administration can elevate the concentration of IL-10 (p<0.01). The results illustrate that UTI inhibits proinflammatory cytokines (TNF-α), which have inhibitory effects on the monocytes and macrophages, and promote the increase of the IL-10. Urinary trypsin inhibitor can restrain excessive expression of iNOS. A small quantity of NO has been shown to be critical to normal physiology maintaining tissue microcirculation and endothelial integrity, whereas excessive NO has been shown to play a major role in the pathogenesis of MODS in septic shock.[17,18] Animal experiments suggest that inhibiting generation of NO can decrease mortality

rates of CLP rats,[19] indicating that keeping NO low ameliorates organ function in sepsis. In septic shock, increased NO production occurs due to an excessive induction of iNOS, which is one of the key cellular responses to sepsis, leading to cell death and tissue toxicity.[20,21] The iNOS plays a crucial role in septic shock[22,23] because iNOS specific inhibitors protected against organ and tissue injury induced by CLP.[24]

In conclusion, that iNOS-derived NO may be responsible for the observed tissue injury after CLP. We have shown that iNOS mRNA expression in lung and liver tissues increased significantly after induction of CLP ($p<0.01$), whereas treatment with UTI inhibited expression of iNOS mRNA after CLP. Moreover, because of the key role of iNOS in this impairment, we suspect that the observed protective effect of UTI includes its inhibitory effect on iNOS induction. Meanwhile, in 50u/g UTI group demonstrated better protective effects than the 20u/g UTI group in all experimental indexes. These results indicate that the protective effects of UTI in sepsis exhibit dose-dependent effect. Based on these results, we conclude that intervention with UTI seemed to have a beneficial antiseptic shock effect at an appropriate dose. Its action mechanisms are probably involved in the inhibition of inflammatory factor production and suppression of iNOS mRNA expression and lipid peroxidation. Additional experiments are required to further determine the different time points of UTI treatment after CLP. It is necessary and valuable to explore further research in these fields.

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