Protective Effects of Ulinastatin Combined With Thrombomodulin Against Lipopolysaccharide Induced Liver and Kidney Injury in Septic Rats

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Abstract

Background: Sepsis, a systemic inflammatory disease that leads to life-threatening organ functions disorders, such as liver and kidney injury. Ulinastatin (UTI) and Thrombomodulin (TM) are active macromolecules isolated from human urine. UTI and TM have been found to have therapeutic effects on inflammatory diseases. In this study, we verified protective effect of UTI combined with TM on liver and kidney injury caused by sepsis, and further explored the mechanisms.

Methods: The sepsis model was established by intravenous injection of LPS into the tail vein of rats. Blood, liver and kidney tissues were collected after injection of UTI or TM. ELISA was used to measure serum levels of pro-inflammatory cytokines. The characteristic functional indexes of liver and kidney in serum and multiple coagulation function indexes of rats were detected via corresponding kits. Histological changes of liver and kidney tissues were investigated by HE staining. Apoptosis in liver and kidney tissues were examined by TUNEL staining, and the expression levels of apoptosis-related proteins were also analyzed. HMGB1/TLR4/NF- κ B pathway in liver and kidney tissues were examined by Western Blot. PCNA-positive cells were detected by immunohistochemistry. The survival rate of rats in each group was statistically analyzed.

Results: UTI combined with TM reduced LPS-induced secretion of IL-6 and TNF- α in the serum. The drug combination reduced the liver and kidney functional indicators ALT, AST, BUN and Cr, and ameliorated liver and kidney pathology injury of rats. It inhibited apoptosis of liver and kidney cells via down-regulating the expression of apoptotic protein Bax, Cleaved caspase-3, up-regulating the expression of anti-apoptotic protein bcl-2, and promoted the proliferation of liver and kidney cells. The drug combination reversed the up-regulation of HMGB1, TLR4, and phosphorylated NF- κ B protein mediated by LPS. Anticoagulation test indicated UTI does not affect the anticoagulant effect of TM when they are used in combination. Moreover, the drug combination significantly improved the survival rate of septic rats.

Conclusions: These results indicate that UTI combined with TM plays a key role in protecting liver and kidney injury in septic rats, which will suggest a promising treatment for sepsis-induced organ injury.

1. Background

Sepsis is a disease with a systemic inflammatory state with known or suspected infection (Vandewalle and Libert. 2020). The mortality rate has remained high for a long time. Pathogenic microorganisms or toxins invade the body through the blood and activate the host immune system (Mirouse et al. 2020), thereby producing endogenous inflammatory mediators and cytokines. At the same time, sepsis affects various systems and organs of the body. It causes damage to cells and tissues and affects metabolism, which further lead to failure of various vital organs (Venet and Monneret. 2018; Tsantarliotou et al. 2019). The liver and kidney are organs vulnerable to damage from sepsis (Yan et al. 2014; Alobaidial. 2015). Acute liver and kidney injury may occur at any stage of sepsis. Sepsis patients usually have severe liver

and kidney injury and often extremely critical, prone to multiple organ failure, leading to high mortality (Cecconi et al. 2018). Therefore, the repair of organ injury is particularly important in the treatment of sepsis.

HMGB1, as a late inflammatory factor, is mainly located in the nucleus and is essential for maintaining cell life. However, when intracellular HMGB1 is actively secreted by activated macrophages and dendritic cells, or passively released from necrotic and apoptotic cells into the outside of the cell, HMGB1 can promote early inflammatory factors such as tumor necrosis factor (TNF)- α and interleukin (IL)-6 release, continuously trigger and maintain the downstream inflammatory response, and participate in the occurrence and development of various diseases including sepsis and autoimmune diseases (Abdulmandi et al. 2017; Magna et al. 2014). In endotoxemia and sepsis, HMGB1 is released into the circulation, which causes high mortality (Lu et al. 2012). HMGB1 mainly activates downstream signaling pathways by binding to its receptors and promotes nuclear metastasis of nuclear factor (NF)- κ B to play its inflammatory regulatory role. TLR4 is one of the main receptors of HMGB1. HMGB1 binds to TLR4 and activates the downstream signaling molecule NF- κ B through the transmembrane signaling pathway (Yang et al. 2020). As a key transcription factor in TLR4-mediated signal transduction, NF- κ B can regulate a variety of factors and signal pathways related to inflammatory response.

Ulinastatin (UTI) is an important endogenous broad-spectrum protease inhibitor extracted and purified from adult male urine. It can effectively inhibit serine protease, which is a key component of inflammatory response (Liu et al. 2015), and play a vital role in the regulation of inflammation in intercellular or intracellular signaling pathways. Protease inhibitors can counter-regulate the expression of proteases during inflammation to inhibit the progression of inflammation (Kessenbrock et al. 2011). Therefore, UTI is considered to have an essential anti-inflammatory effect. UTI is clinically used in the treatment of acute circulatory failure, acute pancreatitis and other diseases. Importantly, UTI reflects a good protective effect on organs and tissues, and at the same time contributes to the treatment of collective infections, tumor diseases, organ and tissue injury, coagulation dysfunction and diabetes caused by various reasons. It is determined that UTI has a protective effect on many organs to prevent various human diseases including sepsis (Song et al. 2019; Cao et al. 2018; Yang et al. 2020).

Thrombomodulin (TM) derives from fresh human urine too, and its recombinant product rhTM has been approved for clinical pancreatitis and diffuse intravascular coagulation (DIC) treatment. The beneficial effect of TM on DIC has been confirmed by plenty of studies. It has been reported that the use of rhTM correlates with the reduction of mortality in DIC adult patients with sepsis. In order to exert its anticoagulant effect, rhTM forms a complex with thrombin to inhibit coagulation activity (Yatabe et al. 2018). The thrombin-rhTM complex itself converts protein C into activated protein C, thereby selectively producing activated factors V (Va) or activated factor inactivates VIII (VIIIa), which contributes to further inhibition of thrombin formation (Pescatore. 2001). Recently, the pleiotropic properties with anti-inflammatory effects of TM have attracted people's attention (Ma et al. 2015). Existing studies have shown that recombinant TM and its D1 reduce the level of histone H3 and thereby alleviating the acute kidney injury caused by sepsis (Akatsuka et al. 2020). In addition, it can effectively protect the kidney

from injury. In the field of liver injury caused by sepsis, the administration of recombinant TM can also improve the liver dysfunction and elevate the survival rate of septic mice (Nagato et al. 2009). In a retrospective study, recombinant human soluble TM can decrease the mortality of patients with sepsis (Yoshihiro et al. 2019). These all indicate that TM can be used in the treatment of sepsis, and has a broad research prospect in organ injury, but its specific organ protection mechanism remains unclear.

In previous studies, both UTI and TM have been found to have therapeutic effects on sepsis (Jiang et al. 2018; Wu et al. 2019; Wang et al. 2019; Ashina et al. 2020), and more meaningfully, a number of studies have shown that UTI combined with other drugs can effectively improve the treatment effect of sepsis (Meng et al. 2020; Wang et al. 2020). At present, there are many research projects on sepsis, but the research based on drug combination therapy is not sufficient. In summary, we use UTI combined with TM to verify its protective effect on liver and kidney injury caused by sepsis, and further explore the mechanisms of action.

2. Materials And Methods

2.1 Animals and Treatments

Male wistar rats at the age of 7–9 weeks weighing 200–220 g were obtained from the Experimental Animal Center of Yangzhou University. The rats were held in specific pathogen-free conditions in Animal Experiment Center of China Pharmaceutical University for at least one week prior to commencing studies. They were housed in a 12 h light–dark cycle-controlled room, and set at room temperature at 23–25°C and humidity (40–70%), and fed with standard laboratory diet and water. All healthy rats were randomly divided into five groups (n = 12). These groups were established as followed: Rats from Sham group received normal saline injection; rats from LPS group received LPS (10 mg/kg Escherichia coli, 055: B5, Sigma, MO, USA) injection; rats from UTI group received LPS and UTI (50000 U/kg, Adeal, Yangzhou, Jiangsu, China) injections; rats from TM group received LPS and TM (2000 U/kg, Adeal, Yangzhou, Jiangsu, China) injections; rats from UTI + TM (UTI 50000 U/kg + TM 2000 U/kg) group received LPS, UTI and TM injections. Throughout the experiment, 1 h after LPS injection, UTI, TM or UTI + TM were injected. LPS, UTI and TM were injected into the rat body via tail vein. In addition, 60 rats were grouped as described above for later survival analysis (n = 12). The protocol was approved by the Animal Research Committee of China Pharmaceutical University.

2.2 Blood, liver and kidney tissue preparation

At 6, 12, 24 hours after the injection of drugs, 5 mL of blood was collected from the orbital vein of 60 rats and centrifuged at 3000 g for 15 minutes. The supernatants were collected and store at – 80°C for further use. Rats were killed 24 hours after drugs injection to harvest liver and kidney for subsequent experiments.

2.3 Enzyme-linked immunosorbent assay (ELISA)

To monitor the degree of inflammatory response of each group, serum collected at 6 h after drugs injection was used. Serum levels of IL-6 and TNF- α were measured by using the ELISA kits (MEILIAN, Shanghai, China) according to the manufacturer's protocol. The reaction plates were read within 15 minutes in an ELISA plate reader (Thermo Fisher, Vantaa, Finland) at 450 nm. TNF- α and IL-6 concentrations were calculated relative to the appropriate standard curve and expressed as pg/ml.

2.4 Biochemical analysis

The characteristic functional indicators of liver and kidney in each group were tested, and serum was taken at a 24 h time point. Blood urea nitrogen Kit (C013-2-1 Jiancheng, Nanjing, China), creatinine (Cr) Determination Kit (C011-2-1, Jiancheng, Nanjing, China), Aspartate aminotransferase (AST) Assay Kit (C010-2-1 Jiancheng, Nanjing, China), and Alanine aminotransferase (ALT) Assay Kit (C009-2-1, Nanjing Jiangcheng, Nanjing, China) were used to assess the activity of serum BUN, Cr, AST and ALT of each group.

2.5 Haematoxylin and Eosin (HE) staining

Slices of liver and kidney were fixed for 48 h in 10% neutral buffered formalin, then dehydrated in graded concentrations of ethanol and embedded in paraffin. Samples were sectioned at 5 μ m thick for HE staining. The liver and kidney injury was observed under an optical microscope with x 400 magnification (Nikon, Japan) and then photographed. Pathologists perform independent quantification scores for each specimen through blind.

2.6 Terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) assay

Kidney and liver tissues embedded in paraffin were sliced at 5 μ m thick for TUNEL assay. The TUNEL method (KGA7071, Keygen, Nanjing, China) was used to detect cell apoptosis in liver tissues according to the manufacturer's instructions. Finally, DAPI (KGA215, Keygen, Nanjing, China) was used for mounting, and positive cells in the field of view were observed under an optical microscope with x 400 magnification (Nikon, Japan).

2.7 Immunohistochemical analysis of proliferation of cell nuclear antigen (PCNA)

The procedure for paraffin sectioning of liver and kidney was as described above. Paraffin sections were washed for antigen retrieval, and treated with enzymatic inactivation. Then they were blocked with 1% BSA50, incubated with the primary antibody overnight at 4 °C. Goat anti-rabbit polymer (ab92552, ABCAM) was added and incubated for 20 minutes. DAB solution (DAB-1031, Xinmai, Fuzhou, China) was added for color development. Hematoxylin was continually added until complete dehydration and mounting, and finally complete dehydration and mounting. The percentage of 400 x PCNA positive cells was analyzed in 5 random fields in each section.

2.8 Western blot

Western blot was used to detect the expression of protein. Membranes were blocked with 5% TBST diluted non-fat powdered milk at room temperature for 1 hour incubated with primary antibody (HMGB1, sc-56698, santa cruz; TLR4S, sc-293072 santa cruz; P-NF- κ B p65, sc-166748 santa cruz; Cleaved caspase3, #9961, Cell Signaling Technology; NF- κ B p65 #8242, Cell Signaling Technology; bcl-2, WL01556, Wanlei; Bax WL01637 Wanlei; GAPDH WL01114; β -actin WL01372 Wanlei.) at 4 °C for 14 h. β -actin and GAPDH were used as the standard proteins. The secondary antibody was diluted with blocking solution and incubated membranes for 90 mins at room temperature. Image J was used to analyze the gray value of the protein band.

2.9 Survival

After drugs injection, the survival rate of rats in control group, LPS group, TM group, UTI group and UTI + TM group were recorded, and the whole statistics lasted for 7 days.

2.10 Rat plasma collection and processing

After fasting overnight, blood was taken from the artery of rats and plasma was prepared. All plasma samples were tested within 2 h at room temperature to ensure the stability. TM or UTI dissolved in physiological saline solution were mixed with rat plasma in a volume ratio of 1 : 99. The mixture was conducted to explore the effects of TM or UTI + TM on rat plasma coagulation indicators. At the same time, a blank control group was set. The blank control group was prepared by mixing physiological saline with rat plasma at a volume ratio of 1 : 99.

2.11 Coagulation index detection

The thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT) detection kits (Sun, shanghai, China) were used to test these indicators using a semi-automatic coagulation factor analyzer (Zhongqingshidi, Taizhou, China). The instrument parameters were set, and stirring beads were added to each channel of the test cup and the drug-containing plasma to be tested (TT: 100 μ L, PT: 50 μ L, APTT: 50 μ L and APTT reagent 50 μ L) into the test cup, placed in the 37 °C pre-warming zone for 3 minutes. The test cup was transferred to the test zone, and detection reagents were added (TT: TT solution 100 μ L, PT: PT solution 100 μ L, APTT: CaCl_2 solution 50 μ L). Then, the results could be recorded after measuring various coagulation indexes.

2.12 Statistical analysis

All data were presented as mean $\pm$ standard deviation (SD). SPSS version 23.0 (IBM SPSS Statistics) and GraphPad Prism 8.0 (GraphPad Software) were used to analyze and graph all data. One-way analysis of variance (ANOVA) followed by post hoc Tukey test were used to assess the significance of statistical differences between groups. A P-value < 0.05 was considered statistically significant.

3. Results

- **3.1 UTI combined with TM reduced LPS-induced secretion of IL-6 and TNF- α in the serum of rats**
- LPS-induced sepsis lead to the secretion of pro-inflammatory cytokines (including IL-6 and TNF- α) in serum, we assessed the effect of the combination of UTI and TM on the release of inflammatory factor TNF- α and IL-6, and the systematic immune status of rat after LPS injection. ELISA analysis results showed that, compared with the sham group, LPS could significantly induced the increase in serum IL-6 and TNF- α . Both UTI and TM treatment alone reduced the serum content of IL-6 and TNF- α , but the combination of TM and UTI further reduced the levels of IL-6 and TNF- α induced by LPS (Fig. 1.A), (Fig. 1.B).

3.2 The effect of UTI combined with TM on the characteristic indexes of liver and kidney in rats

ALT and AST are two vital biomarkers of liver injury. Results of serum test showed that after the injection of LPS, compared with sham group, ALT and AST levels of the model group increased sharply, which confirmed that the liver function of the model group was hit and damaged. 24 h after using TM and UTI, the serum ALT and AST values of rats decreased. In UTI + TM group, ALT and AST values were further reduced (Fig. 2.A), (Fig. 2.B), indicating that UTI combined with TM effectively restored liver function injury. The same trend was also observed in renal function indicators. Compared with the sham group, the serum renal biomarkers BUN and Cr levels of the LPS group increased significantly, while the BUN and Cr values decreased in TM group and UTI group. The BUN and Cr values of drug combination group further downregulated (Fig. 2.C), (Fig. 2.D), indicating that UTI combined with TM also effectively restored the renal function injury in septic rats.

3.3 UTI combined with TM reduced liver and kidney pathology injury

HE stained liver sections showed that the model group injected with LPS caused obvious liver injury, in which the liver tissue cells were irregularly arranged, accompanied by a large number of necrosis and inflammatory cell infiltration. The liver in sham group did not suffer injury. In both TM group and UTI group, these changes were alleviated to a certain extent, the cells were clear and inflammatory cell infiltration was seen in the portal area. However, in UTI + TM group, the liver injury was further reduced, the liver cells were clear, regularly arranged, there was no obvious edema, and the necrosis was reduced (Fig. 3.A). Kidney HE stained section, it can be observed that after injection of LPS, although the glomerular structure is normal, there are renal tubular swelling and vacuolar degeneration and necrosis of epithelial cells. A large number of inflammatory cell infiltration can be seen in the renal interstitium. The renal tubules of UTI group and TM group were still swollen, but the vacuolar degeneration of renal tubular epithelial cells was reduced, and the interstitium was infiltrated by inflammatory cells. The pathological changes in drug combination group were further improved. The swelling was further reduced, the vacuolar degeneration of renal tubular epithelial cells was reduced, and only a small amount of inflammatory cell infiltration was observed (Fig. 3.B).

We also scored histological damage on HE stained sections. The higher the score displayed in the histogram, the more severe the tissue damage. A score of 0 in the sham group means that there is no damage to the liver and kidney tissues. The liver and kidney scores of the LPS group are all greater than 2.5, which means that the tissue damage is severe, liver and kidney histology scores of UTI group, TM group and drug combination group were all reduced, but drug combination group had the lowest score (Fig. 3.C), (Fig. 3.D), which indicated that the combination of drugs has a stronger effect on the recovery of damaged tissues than UTI or TM treatment alone.

3.4 UTI combined with TM inhibited apoptosis of liver and kidney in septic rats

In order to clarify the protective mechanism of UTI combined with TM on liver and kidney injury in septic rats, TUNEL staining was used to observe the apoptotic cells in liver (Fig. 4.A) and kidney (Fig. 4.B) slides. Compared with sham group, positive cells in the LPS group were significantly increased, apoptosis was more likely to occur, while either UTI or TM treatment reduced the positive cells. In UTI + TM group, the number of apoptotic cells further decreased. We also performed statistical analysis on the positive rates of liver (Fig. 4.C) and kidney (Fig. 4.D) cells in TUNEL staining. Whether in liver or kidney tissue, the apoptosis rate of LPS group was increased significantly, while after the administration of UTI and TM, the apoptosis rate was appear to decreased, and the apoptosis rate of the combination group further decreased. In addition, beside the results of TUNEL analysis, UTI and TM treatment reduced the apoptotic protein Bax and Cleaved caspase-3. When UTI and TM were used in combination, this downward trend was further accentuated, otherwise LPS will up-regulated the expression of Bax and cleave caspase-3. The decrease in the anti-apoptotic protein bcl-2 in LPS group was be reversed by the combination of UTI and TM (liver: Fig. 5.A-D; kidney: Fig. 5.E-H).

These data indicated that the combination of UTI and TM can protect the liver and kidney by reducing the expression of pro-apoptotic proteins, thereby reducing cell apoptosis.

3.5 Immunohistochemistry of PCNA

To investigate the effects of TM and UTI on the proliferation of hepatocytes and kidney cells, we performed immunohistochemical analysis on PCNA (liver: Fig. 6.A), (kidney: Fig. 6.B). We counted the number of hepatocytes in randomly taken photomicrographs. Compared with sham group, the ratio of positive hepatocytes/total hepatocytes decreased significantly after injection of LPS. Compared with LPS group, in UTI group, the positive rate increased by 3.71%, in TM group by 4.21%, the positive rate in UTI + TM group further increased by 7.39%. During the regeneration process, the proportion of positive hepatocytes of UTI + TM group was greater than that of TM group and UTI group (Fig. 6.C). We also analyzed the PCNA of the kidney. During the whole process, the changes of PCNA in the kidney of each group of rats were consistent with the expression of PCNA in the liver. Compared with sham group, the ratio of positive kidney cells/total kidney cells decreased. Compared with LPS group, in UTI group, the ratio increased by 2.68%, in UTI group by 4.60%, and further increased by 6.36% in UTI + TM group (Fig. 6.D).

3.6 UTI combined with TM inhibited TLR4-mediated NF-κB pathway

TLR4 has been identified as a receptor for LPS, and TLR4-related signal transduction pathways may mediate liver and kidney injury in septic rats. In this study, in order to explore the potential mechanism of UTI combined with TM in protecting liver and kidney injury in septic rats, the expression of HMGB1, TLR4 and the phosphorylation of NF-κB in liver and kidney tissues were evaluated. As shown in Fig. 7 (liver: Fig. 7.A-D), (kidney: Fig. 7.E-H), compared with relative to the expression of the endogenous control β-actin, HMGB1, TLR4, and P-NF-κB proteins all increased significantly in liver and kidney tissues in LPS group. The administration of UTI and TM remarkably inhibited the protein expression of HMGB1, TLR4 and P-NF-κB. As expected, the protein expression of HMGB1, TLR4 and P-NF-κB further decreased in septic rats administered TM and UTI in combination. UTI combined with TM also significantly reduced the protein expression of downstream NF-κB.

3.7 Influence of UTI on the anticoagulant function of TM

As shown in Table.1, as expected, in the range of 0–200 U/mL, TT, PT and APTT upregulated with the increase of TM concentration, and all had a good linear correlation ($r^2 > 0.98$). TT was extremely sensitive to TM. Compared with the blank control group, 200 U/mL TM significantly prolonged TT by about 3.23 times ($P < 0.05$). However, the increase of UTI concentration in the range of 0–200 U/mL did not extend the TT, PT and APTT of rat plasma ($P < 0.05$, there was no statistical difference in anticoagulant effect). According to the above results, 100 U/mL of TM was selected for subsequent experiments with UTI (100 U/mL) to investigate whether UTI would reduce the anticoagulant effect of TM when combined. Importantly, when UTI was used in combination with TM, TT, PT, and APTT were not shortened compared with TM group, which indicated that UTI does not affect the anticoagulant effect of TM when they are used in combination (Table.2).

3.8 UTI combined with TM improved the Survival Rate of LPS-Induced septic Rats

The survival period of rats was monitored every 24 hours for 7 days. The rats in Sham group showed no abnormal behavior and no death was observed. However, after inject of LPS, rats developed diarrhea, lethargy and ruffled pelage. The number of surviving rats in LPS group decreased linearly with time, which was significantly lower than in Sham group ($P < 0.05$). UTI and TM were administered separately to improve the survival rate of septic rats. When UTI and TM were used in combination, the survival rate of rats had a further improvement trend (Fig. 8). Compared with the single-drug group, UTI + TM treatment increased the average survival rate by 33.3% ($n = 12$).

4. Discussion

Sepsis is a type of systemic inflammation response syndrome, which has the characteristics of rapid disease progression and high case fatality rate. It has always been one of the difficulties in critical illness research. Society of Critical Care Medicine (SCCM) and European society of intensive medicine (ESICM) newly defined sepsis as the unbalanced response of the body to infection that leads to life-threatening organ functions disorders (Vincent et al. 2016; Singer et al. 2016). The old definition of sepsis is a systemic inflammatory response syndrome caused by infection, which emphasizes infection, while the new definition of sepsis focuses on the body's response to infection and imbalance with organ dysfunction. This definition suggests that more attention should be paid to the complex pathophysiological response caused by infection during treatment. It is a return to the understanding of the essence of sepsis. Therefore, in the treatment of sepsis, protecting organs from damage and repairing organ injury is particularly important. Most organ injury in sepsis is caused by the inflammatory response involving excessive and dysfunctional cytokines, and the effects of the existing treatment strategies for sepsis-induced organ injury are not satisfactory. In consequence, there is an urgent need for new therapies or drugs for sepsis. UTI and TM are glycoproteins extracted from human urine. Several studies have reported that these two drugs have clear anti-inflammatory effects and a certain protective effect on liver and kidney injury. Therefore, we further tested the protective effect of UTI combined with TM on sepsis.

LPS is a component in the outer wall of gram-negative bacteria (Cavaillon. 2017). It is a classic inducer of sepsis in medical research (de Pádua et al. 2018). It can activate mononuclear macrophages and endothelial cells through the cell signal transduction system in the body, synthesize and release a variety of inflammatory mediators (Plociennikowska et al. 2015), which in turn cause a series of reactions to the body. Pro-inflammatory cytokines such as IL-6 and TNF- α are involved in the initiation and regulation of the inflammatory response (Firinu et al. 2016). Reliable studies have confirmed that a large amount of TNF- α and IL-6 are produced in macrophages exposed to LPS (Lee et al. 2017). In our research, Serum pro-inflammatory factors TNF- α and IL-6 were significantly increased in rats injected with LPS. However, UTI combined with TM significantly inhibited the levels of TNF- α and IL-6 in serum, indicating that its protective effect on sepsis may be related to its anti-inflammatory properties.

As a kind of endotoxin, LPS is directly injected into the blood by intravenous injection to cause sepsis in rats. animals infused with LPS manifest features of compensated human sepsis, including hypotension, hypermetabolism and elevated serum lactate concentrations, this model can be used to study mechanisms that contribute to the activation of inflammatory cascades induced by bacterial antigen, and unravel interactions between distinct inflammatory systems (such as coagulation system and cytokine network), and to clarify the principle for the efficacy of novel anti-inflammatory compounds (Fiuza et al. 2001; Lowry. 2005; Van der Poll. 2012). In addition, the infectious dose of this model can be controlled according to the weight of the rats, and it has good stability, repeatability and controllability (Yeh et al. 2016). This method is currently widely used (Hao et al. 2017; Carty. 2019; Sun. 2018; Savio et al. 2017).

HMGB1/TLR4/NF- κ B is an important inflammatory signal pathway in LPS-induced inflammation (Shang et al. 2019). Some reports have shown that HMGB1/TLR4 common syndrome pathway genes are

expressed in the liver and kidney (Liu et al. 2020; Mohamed et al. 2020). HMGB1 is a highly conserved non-histone DNA binding protein, which is widely distributed among various organs such as lung, brain, liver, heart, and kidney. HMGB1 can be released from necrotic cells through active secretion and passive release, inducing inflammation. HMGB1 is also one of the endogenous ligands of TLR4, which also widely expresses in liver and kidney. LPS induces tissues to release HMGB1, which mediates autophagy or triggers the initiation of inflammation through the TLR4 signaling pathway, triggering a series of cascade reactions. It mainly includes two pathways, including myeloid differentiation factor 88 (MYD88) dependent pathway and TRIF dependent pathway (Wang et al. 2015). Activation of MYD88 activates downstream IKK- α /IKK- β , leading to phosphorylation and degradation of I κ B- α , and finally NF- κ B is activated (Zhang et al. 2018). Phosphorylation of NF- κ B leads to the release of pro-inflammatory cytokines, including TNF- α , IL-1 β and IL-6. Therefore, inhibiting HMGB1-TLR4 signaling pathway may effectively improve organ injury caused by sepsis. Our research have confirmed that UTI and TM can significantly inhibit LPS-induced liver and kidney injury through the HMGB1-TLR4-NF- κ B pathway. When UTI and TM were used in combination, this effect was more significant, and it also upregulated the survival rate of rats attacked by LPS.

It is worth noting that LPS can promote apoptosis of liver and kidney cells and aggravate tissue injury (Zhang et al. 2020; Lu et al. 2020). It was verified by western blot that the protein concentration of Cleaved caspase-3 and Bax increased, and the concentration of anti-apoptotic protein bcl-2 decreased. In serum, ALT and AST, as indicators of liver characteristics, and as well as BUN and Cr, as indicators of kidney characteristics, were both increased. It showed that the action of endotoxins led to apoptosis of liver cells and kidney cells. With the administration of UTI and TM, the concentration of Cleaved caspase-3 and Bax decreased, the concentration of bcl-2 increased, and the values of ALT, AST, BUN and Cr began to decrease. All this indicates that liver and kidney injury were alleviated. When UTI combined with TM, this protective effect was better. This result was further verified in the TUNEL experiment, the results were further verified. The number of positive cells in liver and kidney tissues decreased after UTI combined with TM, which reversed the increase in the number of positive cells caused by LPS. We also observed the changes in the number of PCNA-positive cells in the liver and kidney tissues. The changes in PCNA were usually closely related to tissue regeneration (Lee et al. 2019). After LPS injection, the PCNA of rat liver and kidney tissues decreased significantly. LPS exposure would affect these proliferating cells and cause the decrease of their number due to cell death. After the administration, PCNA began to increase, and the combination group's level was higher than LPS group. Interestingly, we observed that at the concentration we set, the single-drug groups increased in proliferation, but not significantly compared to LPS group. This indicated that the combination of UTI and TM may have a limited effect on promoting regeneration, in contrast, inhibiting apoptosis has a stronger protective effect.

The blood coagulation system plays an important role in the pathogenesis of sepsis. It promotes each other with inflammation, and together constitutes a key factor in the occurrence and development of sepsis (Levi et al. 2010). Endotoxin can activate the exogenous coagulation pathway by inducing the release of tissue factor of macrophages and endothelial cells. The coagulation factor XII activated by endotoxin can also further activate the endogenous coagulation pathway, which ultimately leads to

diffuse DIC (Park et al. 2016; Latour. 1983). Therefore, we envision that in the treatment of DIC induced by sepsis, TM can not only effectively treat sepsis, but may inhibit the symptoms of DIC by activating the anticoagulation system. Our experiments proved that compared to using TM alone, UTI combined with TM did not reduce the anticoagulant effect of TM. We concluded that combined use of UTI and TM was effective in the treatment of sepsis, and UTI did not affect the anti-DIC effect of TM. Therefore, when choosing medication in such special populations of DIC induced by sepsis, UTI and TM combined administration is a good strategy.

It is undeniable that this study still has certain limitations. First, our experiments did not conduct further studies into the damage and repair of other important organs such as the heart and lungs. In addition, existing studies have shown that UTI and TM have multiple pharmacological effects and may also trigger other mechanisms, the protective mechanism of drug combination on liver and kidney is worthy of further exploration.

5. Conclusion

5.Conclusions

In conclusion, our research showed that the combined use of UTI and TM protected liver and kidney of LPS-treated rats. These protective effects can be attributed to UTI and TM reducing certain inflammatory mediators such as TNF- α , IL-1 β and IL-6, inhibiting apoptosis and HMGB1/TLR4/NF- κ B signaling, and promoting the proliferation of liver and kidney cells (Fig. 9). The protective effect of septic liver and kidney injury and inflammation provided new insights into the treatment of liver and kidney injury caused by sepsis. Therefore, considering these results, the combination of TM and UTI may be a promising strategy for the treatment of sepsis.

Abbreviations

UTI Ulinastatin

TM Thrombomodulin

DIC Diffuse intravascular coagulation

LPS Lipopolysaccharide

TNF Tumor necrosis factor

IL Interleukin

NF Nuclear factor

HMGB1 High mobility group protein 1

BUN Blood urea nitrogen

Cr Creatinine

AST Aspartate aminotransferase

ALT Alanine aminotransferase

HE Haematoxylin and Eosin

TUNEL Terminal-deoxynucleotidyl transferase mediated nick end labeling

PCNA Proliferation of cell nuclear antigen

TT Thrombin time

PT Prothrombin time

APTT Activated partial thromboplastin time

SCCM Society of Critical Care Medicine

ESICM European society of intensive medicine

MYD Myeloid differentiation factor

Declarations

Ethics approval and consent to participate

Our experiment complies with the Basel Declaration, which outlines the basic principles to be followed when conducting research in animals. The protocol was approved by the Animal Research Committee of China Pharmaceutical University.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

FY and JL designed experimental scheme and prepared the manuscript. XZ and CS carried out the experiment. XZ and EE wrote the paper. JY and FY revised the manuscript critically and supplemented the experiment. XL and CF performed experimental data analysis.

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Tables

Table.1 Effects of UTI and TM on coagulation indicators of rat plasma.

Indicator	c/(U/mL)	TT/s	PT/s	APTT/s
UTI	0	41.52±0.66	18.10±0.29	37.75±0.58
	50	41.52±0.66	18.10±0.29	37.75±0.58
	100	41.33±1.25	18.85±0.46	38.18±0.94
	150	42.17±0.66	18.83±0.65	39.35±0.77
TM	0	41.52±0.66	18.10±0.29	37.75±0.58
	50	81.57±1.14*	22.03±0.52*	51.32±0.97*
	100	114.23±1.97*	24.02±0.59*	69.33±1.07*
	150	147.10±2.18*	29.18±0.62*	79.45±1.31*

*P<0.05 vs. 0 µg/mL group. TT: thrombin time; PT: prothrombin time; APTT: activated partial thrombin time

Table. 2 After combined use of UTI, the effect on anticoagulation index of TM

Group	c/(U/mL)	TT/s	PT/s	APTT/s
Control	0	41.52±0.66	18.10±0.29	37.75±0.58
UTI	100	42.17±0.66	18.83±0.65	39.35±0.77
TM	100	114.23±1.97*	24.02±0.59*	69.33±1.07*
UTI+TM	100+100	119.21±3.31*	24.20±0.82*	70.78±0.87*

*P<0.05 vs. Control group. TT: thrombin time; PT: prothrombin time; APTT: activated partial thrombin time

Figures

Figure 1

Changes of serum cytokines in rats after LPS injection or sham by ELISA kit. (A) Changes in five groups of TNF-α; (B) Changes in five groups of IL-6. * $P < 0.05$ vs. UTI group. # $P < 0.05$ vs. TM group, $\Delta P < 0.05$ vs. Sham group, $\square P < 0.05$ vs. LPS group.

Figure 2

Changes of serum ALT, AST, BUN and Cr levels in rats. (A) serum ALT; (B) serum AST; (C) serum BUN; (D) serum Cr. * $P < 0.05$ vs. UTI group. # $P < 0.05$ vs. TM group, $\Delta P < 0.05$ vs. sham group, $\square P < 0.05$ vs. LPS group.

Figure 3

Histological changes in liver and kidney tissues of LPS induced septic rats. (A) Representative pictures of HE staining of liver tissues in each group, the black arrows point to the inflammatory cells; (B) Representative pictures of HE staining of kidney tissues in each group, the black arrows point to the renal tubular swelling; (C) Score of liver tissues injury in five groups; (D) Score of kidney tissues injury in five

groups. Score is proportional to the severity of tissues injury. * $P < 0.05$ vs.UTI group. # $P < 0.05$ vs. TM group, $\Delta P < 0.05$ vs. sham group, $\boxtimes P < 0.05$ vs. LPS group.

Figure 4

Apoptosis in liver and kidney tissues were examined by TUNEL staining. Nuclei were counterstained with DAPI (blue) and DNA fragmentation was measured by TUNEL (+) (green), the red arrows point to the apoptosis cells. (A) Representative images of TUNEL performed on liver slices in five groups; (B) Representative images of TUNEL performed on kidney slices in five groups; (C) Quantification of TUNEL-positive liver cells in sections of each groups; (D) Quantification of TUNEL-positive kidney cells in sections of each groups. $\Delta P < 0.05$ vs. sham group, $\boxtimes P < 0.05$ vs. LPS group.

Figure 5

Measure the expression levels of apoptosis-related proteins in liver and kidney tissues by Western Blot. (A) Protein levels of Bax, bcl-2 and Cleaved-caspase3 in liver tissues; (B) Protein levels of Bax, bcl-2 and Cleaved-caspase3 in kidney tissues; (C) Densitometric analyses of Bax protein in liver tissues; (D) Densitometric analyses of bcl-2 protein in liver tissues; (E) Densitometric analyses of Cleaved-caspase3 protein in liver tissues; (F) Densitometric analyses of Bax protein in kidney tissues; (G) Densitometric

analyses of bcl-2 protein in kidney tissues; (H) Densitometric analyses of Cleaved-caspase3 protein in kidney tissues. GAPDH was used as the control to confirm equal protein loading. *P<0.05 vs.UTI group. #P < 0.05 vs. TM group, Δ P < 0.05 vs. sham group, $\square$ P < 0.05 vs. LPS group.

Figure 6

Evaluation of cell proliferation in liver and kidney tissues Immunohistochemistry was used to detect PCNA-positive cells in liver and kidney tissues. The yellow arrows point to PCNA-positive cells. (A)

Representative images of PCNA of liver tissues; (B) Representative images of PCNA of kidney tissues; (C) Statistics of the positive rate of PCNA in the liver tissues; (D) Statistics of the positive rate of PCNA in the kidney tissues; *P<0.05 vs.UTI group, ΔP < 0.05 vs. sham group, □P < 0.05 vs. LPS group.

Figure 7

Effect of UTI combination with TM on HMGB1/TLR4/NF-κB pathway-related proteins in liver and kidney tissues. (A) Protein levels of HMGB1, TLR4 and NF-κB in liver tissues; (B) Protein levels of HMGB1, TLR4

and NF-κB in kidney tissues; (C) Densitometric analyses of HMGB1 protein in liver tissues; (D) Densitometric analyses of TLR4 protein in liver tissues; (E) Densitometric analyses of p- NF-κB protein in liver tissues; (F) Densitometric analyses of HMGB1 protein in liver tissues; (G) Densitometric analyses of HMGB1 protein in kidney tissues; (H) Densitometric analyses of TLR4 protein in kidney tissues; (I) Densitometric analyses of p- NF-κB protein in kidney tissues.β-actin was used as the control to confirm equal protein loading. *P<0.05 vs.UTI group. #P < 0.05 vs. TM group, ΔP < 0.05 vs. sham group,⊠P < 0.05 vs. LPS group.

Figure 8

The effect of UTI combination with TM on the number of the survival rats. n =12 in survival rate evaluation. ΔP < 0.05 vs. sham group, ⊠P < 0.05 vs. LPS group.

Figure 9

UTI combined with TM protected liver and kidney injury caused by sepsis. UTI combined with TM reversed the outbreak of serum inflammatory factors (IL-6 and TNF- α), reduced serum ALT, AST, BUN and Cr levels, inhibited liver and kidney cells apoptosis by regulating the protein expression of Bax, Cleaved caspase-3, and Bcl-2, promoted the proliferation of liver and kidney cells, and inhibited the signal transduction ability of HMGB1/TLR4/NF- κ B in liver and injury tissues, which is useful in treating liver and kidney injury due to sepsis.