

EFFECTS OF URINASTATIN ON T CELL IMMUNOLOGICAL FUNCTION IN A MURINE MODEL OF SEPSIS

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Abstract

This study aimed to observe the effects of urinastatin (UTI) on the immunological function on a murine model of sepsis. Ninety-six adult CD-SD (Sprague-Dawley) rats were divided into three groups: sham operation group (Sham group), CLP model group (CLP group), and UTI treatment group (UTI group). The murine model of sepsis was prepared by caecal ligation and puncture (CLP) and the survival rate of rats in each group was observed and recorded. The intestinal flora in the faces and the levels of tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6), interleukin-17 (IL-17), plasma endotoxin and the T cell subsets were determined at 6 h, 12 h, 24 h and 48 h after the modelling. The results showed that the levels of TNF- α , IL-6, IL-17, and plasma endotoxin in the CLP group increased significantly compared with the Sham group, indicating that sepsis caused an inflammatory response in the body. After UTI treatment, the levels of TNF- α , IL-6, IL-17, and plasma endotoxin began to decrease, indicating that UTI treatment of sepsis can reduce the highly inflammatory response of the body. Moreover, the overall mortality rate of rats in the UTI group was significantly lower than that of the CLP group. Immune suppression did not occur immediately after CLP in the UTI group, and the CD4⁺ and CD4⁺/CD8⁺ increased with the prolongation of the medication time. In conclusion, UTI can alleviate the excessive inflammatory response, reduce the inflammatory damage, increase the number of CD4⁺ T cells, and improve the state of immune disorder through its anti-inflammatory effect.

Rezumat

Acest studiu a avut ca scop observarea efectelor urinastatinei (UTI) asupra funcției imunologice într-un model murin de sepsis. Nouăzeci și șase de șobolani adulți CD-SD (Sprague-Dawley) au fost împărțiți în trei loturi: grupul de control, grupul de ligatură și puncție cecală (CLP) și grupul tratat cu UTI. Modelul murin de septicemie a fost obținut prin CLP și s-a înregistrat rata de supraviețuire a șobolanilor din fiecare grup. Flora intestinală și nivelurile factorului de necroză tumorală- α (TNF- α), de interleukină-6 (IL-6), de interleukină-17 (IL-17), de endotoxina plasmatică precum și subseturile de celule T au fost determinate la 6 h, 12 h, 24 h și 48 h după inducerea modelului. Rezultatele au arătat că valorile TNF- α , IL-6, IL-17 și endotoxina plasmatică din grupul CLP au crescut semnificativ în comparație cu grupul de control, indicând faptul că sepsisul a provocat un răspuns inflamator în organism. După tratamentul cu UTI, valorile TNF- α , IL-6, IL-17 și endotoxină plasmatică au început să scadă, indicând faptul că tratamentul cu UTI poate reduce răspunsul inflamator. Mai mult, rata generală a mortalității șobolanilor din grupul UTI a fost semnificativ mai mică decât în grupul CLP. Supresia imună nu a apărut imediat după CLP în grupul UTI, iar CD4⁺ și CD4⁺/CD8⁺ au crescut cu durata tratamentului. În concluzie, UTI poate reduce răspunsul inflamator excesiv, leziunile inflamatorii și crește numărul de celule T CD4⁺.

Keywords: sepsis, urinastatin, helper T cell, immunological regulation

Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS) caused by the infection [1]. The pathogenesis of sepsis is very complicated, involving immunity, inflammation and blood coagulation [2]. In the early stage of sepsis, a large number of inflammatory mediators are released, and SIRS is prone to occur [3]. In addition, with the development of the disease, the synthesis and

release of anti-inflammatory cytokines are activated [4] and dominate in the late stage of SIRS. Anti-inflammatory factors suppress the inflammatory response and the immunological function of the cells and organs [5]. These changes cause the body to no longer be able to remove the pathogenic microorganisms of the initial infection effectively, thus, increasing its load [6, 7]. Urinastatin (UTI) is a trypsin inhibitor with anti-

inflammatory and anti-shock effects acting through stabilization of lysosomal membranes and inhibition of hydrolase activity [8]. Thereby, it is commonly used to relieve the organ failure and enhance immunological function in patients with sepsis for clinical treatment [9]. Some studies show that UTI can inhibit polymorphonuclear elastase, TNF- α , proinflammatory cytokines as IL-1, IL-6, and IL-8, protecting the tissues, organs and endothelial cells by the damage caused by sepsis [10, 11]. The number of T lymphocytes and the distribution of each subgroup can reflect the cellular immune state of the body to a certain degree [12]. In this study, we aimed to investigate if UTI administration can protect against sepsis on a rat model and to determine the underlying mechanism of action that has not been completely elucidated till now.

Materials and Methods

Animals

Ninety-six healthy and adult CD-SD rats, 10 - 12 weeks old, weighing between 230 – 250 g, 48 males and 48 females, were selected and randomly divided into three groups according to the random number table method: Sham group (n = 32, 16 males and 16 females), CLP (caecal ligation and puncture) group (n = 32, 16 males and 16 females), and UTI group (CLP + UTI treatment) (n = 32, 16 males and 16 females). Each group was randomly divided into four time-phase sub-groups by random number table method. Rats in the Sham group were divided into 6 h, 12 h, 24 h and 48 h groups after the sham operation, with 8 rats in each group, 4 males and 4 females. The CLP group and UTI group were divided into 6 h, 12 h, 24 h and 48 h groups after the CLP operation, with 8 rats in each group. Rats in different groups were fed in different cages, numbered and recorded separately. The rats for the experiment were placed in an environment with the humidity of $65 \pm 5\%$, and a light-dark cycle of 12 h/12 h. They were free to move and were not restricted in the diet. All rats were kept in the new conditions for one week before the experiment to allow the adaptation to the environment. The protocol of the animal experiment was approved by the ethics committee of The First Affiliated Hospital of Wenzhou Medical University, China, and the experiment also followed the regulations of the State Science and Technology Commission on the management of laboratory animals.

Anaesthesia and skin preparation

Before the experiment, the animals were fasted for 12 hours with free access to water. The rats were anaesthetized by intraperitoneal injection with 0.4 mL of 10% chloral hydrate *per* 100 g body weight. After anaesthesia, the animal was fixed on the operating table in a supine position. The hair of the lower abdominal part was removed initially by surgical bending and shearing.

Establishment of a rat model of sepsis

Caecal ligation and puncture (CLP) method was used as a rat model of sepsis [13]. The rats were placed on the operating table. After anaesthesia, a cut of 1.5 cm on the centre of the abdomen was made to separate the cecum tissue. The terminal 1/2 cecum from the root was ligatured with surgical sutures, and the cecum was pierced twice with a needle to form a caecal leak. A 7-gauge silk thread was kept in place to prevent the needle hole from closing. The abdominal cavity was closed with 1-gauge silk thread, and 10 mL/kg bw 0.9% saline solution was injected immediately after the surgery to prevent the shock. After the surgery, the rat was resuscitated at a constant temperature for 1.5 to 2 hours under a warm lamp. After the rats were awakened, they were allowed to eat and drink freely.

Treatment

Sham group: The animals from the sham group received the same procedure as the sepsis model, the only difference was that after the separation of the cecum and removal from the abdominal cavity, it was put back immediately without other intervention.

CLP group: After CLP modelling, 2 - 3 mL/100 g body weight 0.9 % saline solution (Shanghai Jingke Chemical Technology Co., LTD, China) was injected subcutaneously after the surgery.

UTI group: After CLP modelling, the rats received 1000 U/100 g body weight UTI solution immediately and every 12-hour (4 times in total, at 0 h, 12 h, 24 h and 36 h after modelling). 1000 U/mL UTI solution was prepared from UTI powder (Guangdong Tianpu Bio-pharmaceutical Co. LTD, China) with 0.9% saline solution (Shanghai Jingke Chemical Technology Co., LTD, China). The animals were observed for the first 48 h after surgery and the lethality was recorded.

Detection of intestinal flora

About 0.1 g of rat faeces were collected aseptically based on the time-phase and the experiment groups. The faeces were diluted with 0.9 mL of 0.9% saline solution (Shanghai Jingke Chemical Technology Co., LTD, China). After dilution, the sample was inoculated into faecal coliform culture medium (Nantong Kinghunt Biology Technological Development Co. LTD, China). The concentration of *Escherichia coli*, lactic acid bacteria, and *Bifidobacteria per* gram of faeces was calculated for each group at each time-point.

Blood sample collection and proinflammatory markers determination

The blood was collected separately based on the time-phase and the experiment groups. Two mL of venous blood were collected directly from the tail vein in an EDTA anticoagulation vacuum test tube. The concentration of TNF- α , IL-6 and IL-17 was determined by ELISA method (TNF- α , IL-6 and IL-17 ELISA test kit, Shanghai Shuangying Biological Technology Co., LTD, China). The operation method was strictly in accordance with the instructions attached to the kits. The Kinetic Chromogenic LAL Assays (Hongrongweizai Biological

Engineering Technology Co., Ltd., Shanghai, China) was used to determine the level of plasma endotoxin. The plasma T cell subsets were detected by flow cytometry.

After the cell suspension was oscillated and evenly mixed, the ratio of T cell subsets (CD3⁺, CD4⁺ and CD8⁺) in every 20000 cells of each sample was determined by flow cytometry and the specific value of CD4⁺/CD8⁺ was calculated.

After finishing above sampling and testing 48 hours after modelling, 10 rats from each group (5 males and 5 females) were chosen to be sacrificed. After anaesthesia the animals were sacrificed by exsanguination and used for anatomical observation inside the abdomen.

Statistical analyses

SPSS 19.0 statistical software (IBM, USA) was used for data processing, and analysis of variance and independent sample. Student- T test was used for statistical analysis. All data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and a value of $p < 0.05$ was considered as statistically significant.

Results and Discussion

Clinical observation

The rats in the Sham group awoke earlier after the surgery, and their activities were normal after recovery. Rats in the CLP group awaked with delay after the surgery and suffered from mental depression, vertical hairs, gradual shortness of breath, and bloody excretion in the nostrils and corners of the eyes 10 hours later. The postoperative adverse effects observed in the rats from the UTI group were less in intensity than those in the CLP group. The abdominal cavity was dissected and observed 48 hours after the surgery, and it was

found that there was a small amount of transparent liquid in the abdominal cavity of rat in the Sham group without the smell of stench, obvious signs of exudation, and no bruising swelling at the cecum end. It was basically intact, and the liver was normal. In the CLP group, there was purulent exudation in the laparotomy, and the end of the cecum was black and necrotic partially with a smell of stench. In the UTI group, there was a small amount of bloody exudation in the abdomen, the cecum was dark-green and swollen, and the liver was slightly swollen. Compared with the CLP group the intraperitoneal adverse condition was significantly alleviated in UTI group.

Survival rate of the animals 48 h after surgery

The survival rate of rat in the CLP group was significantly decreased compared with the Sham group ($p < 0.05$). The survival rate in the UTI group was significantly increased compared with the CLP group ($p < 0.05$) (Table I).

The concentration of Escherichia coli, Lactobacillus and Bifidobacterium in faeces

The concentration of *Escherichia coli* in the CLP group was significantly increased compared with the Sham group, while the concentration of *Bifidobacterium* and *Lactobacillus* were significantly decreased compared with the Sham group at all time points ($p < 0.05$). Compared with the CLP group, the concentration of *Escherichia coli* in the UTI group was significantly decreased at 24 h and 48 h after modelling ($p < 0.05$). In UTI group, the concentration of *Bifidobacterium* and *Lactobacillus* were significantly increased at 12 h, 24 h and 48 h after modelling compared with the CLP group (Table II).

Table I

Survival rates of rats in each group 48 hours after the surgery

Group	Total rats in the group	Alive rats 48 h after surgery	Survival rate (%)
Sham group	32	32	100
CLP group	32	11	34.38*
UTI group	32	20	62.50*#

* $p < 0.05$ compared with the Sham group; # $p < 0.05$ compared with the CLP group

Table II

The concentration of *Escherichia coli*, *Lactobacillus* and *Bifidobacterium* in the faeces at different time-points

Group	Time	<i>Escherichia Coli</i> (IgN/g)	<i>Lactobacillus</i> (IgN/g)	<i>Bifidobacterium</i> (IgN/g)
Sham group	6 h	6.44 ± 0.43	8.81 ± 0.47	9.98 ± 0.45
	12 h	6.50 ± 0.38	8.79 ± 0.51	9.09 ± 0.38
	24 h	6.48 ± 0.39	8.75 ± 0.49	10.11 ± 0.52
	48 h	6.55 ± 0.44	8.72 ± 0.65	9.96 ± 0.41
CLP group	6 h	11.82 ± 0.24*	2.16 ± 0.51*	3.27 ± 0.42*
	12 h	12.17 ± 0.52*	1.88 ± 0.43*	2.98 ± 0.26*
	24 h	12.86 ± 0.37*	1.59 ± 0.36*	2.77 ± 0.29*
	48 h	13.51 ± 0.33*	1.35 ± 0.27*	2.50 ± 0.23*
UTI group	6 h	10.16 ± 0.51	3.08 ± 0.36	4.83 ± 0.39
	12 h	9.89 ± 0.42	3.77 ± 0.40#	5.27 ± 0.43#
	24 h	9.56 ± 0.37#	4.25 ± 0.38#	6.05 ± 0.58#
	48 h	9.21 ± 0.43#	4.80 ± 0.36#	6.33 ± 0.37#

* $p < 0.05$ compared with the Sham group at the same time point; # $p < 0.05$ compared with the CLP group at the same time point

The concentration of TNF- α , IL-6, IL-17, and plasma endotoxin in the blood

The concentration of TNF- α , IL-6, and IL-17 in CLP group significantly increased compared with the Sham group ($p < 0.05$). UTI treatment significantly decreased the level of TNF- α , IL-6, and IL-17 compared with CLP group ($p < 0.05$).

The endotoxin concentration was significantly increased in CLP group compared with the Sham group ($p < 0.05$). UTI treatment significantly decreased the endotoxin concentration compared with the CLP group ($p < 0.05$) (Figure 1).

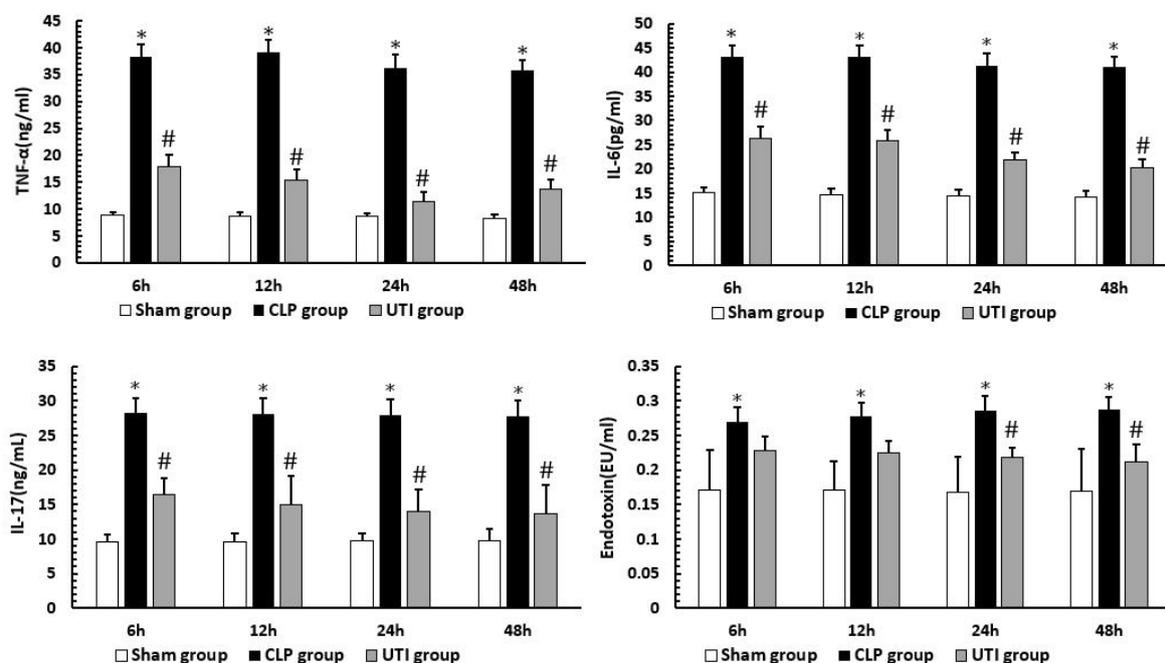


Figure 1.

The concentration of TNF- α , IL-6, IL-17, and plasma endotoxin in the blood of rats of each group
 * $p < 0.05$ compared with the Sham group; # $p < 0.05$ compared with the CLP group

T cell subsets in the blood

There was no statistically significant difference in the proportion of T lymphocyte cell subsets of rats at each time point in the Sham group ($p > 0.05$).

CD3⁺ decreased significantly at 6 h, 12 h, 24 h and 48 h after the surgery in the CLP group compared with the Sham group, ($p < 0.05$). UTI treatment determined a significant increase of CD3⁺ level compared with the CLP group at each time point ($p < 0.05$) and a significant increase compared with the Sham group at 24 h and 48 h after the surgery ($p < 0.05$) (Figure 2).

CD4⁺ decreased significantly at each time point in the CLP group compared with the Sham group ($p < 0.05$). UTI treatment determined a significant increase of CD4⁺ compared with the CLP group at each time point ($p < 0.05$) and a significant increase at 12 h, 24 h and 48 h after the surgery compared with the Sham group ($p < 0.05$) (Figure 2).

CD8⁺ increased significantly at 12 h, 24 h and 48 h after the surgery in the CLP group compared with the Sham group ($p < 0.05$). UTI treatment significantly decreased the CD8⁺ level at 48 h after surgery compared with the CLP group ($p < 0.05$).

CD4⁺/CD8⁺ decreased significantly at each time point in the CLP group compared with the Sham group ($p < 0.05$).

UTI treatment determined a significant increase of CD4⁺/CD8⁺ compared with the CLP group at 12 h, 24 h and 48 h after surgery ($p < 0.05$) and a significant increase at 24 h and 48 h after the surgery compared with the Sham group ($p < 0.05$) (Figure 2).

The immunoregulatory mechanism of sepsis is very complicated. Using drugs such as UTI to improve the immune functions of the organism is the current main treatment method for the treatment of sepsis [14]. Under normal circumstances, there is a balanced proportional relationship between the intestinal floras of rats, and the number of anaerobic bacteria (*Lactobacillus* and *Bifidobacterium*) is higher than the number of aerobic bacteria (*Escherichia coli*) [15, 16]. The results of this study show that the numbers of anaerobic bacteria, *Bifidobacterium* and *Lactobacillus*, in the rat model with sepsis are significantly reduced, and the number of aerobic bacteria, *Escherichia coli*, is increased showing a significant disturbance of the intestinal flora. The UTI can regulate the balance of intestinal floras to a certain extent, prevent disorder of the intestinal flora, promote the microbial barrier of the intestinal mucosa, and prevent the invasion of pathogenic bacteria.

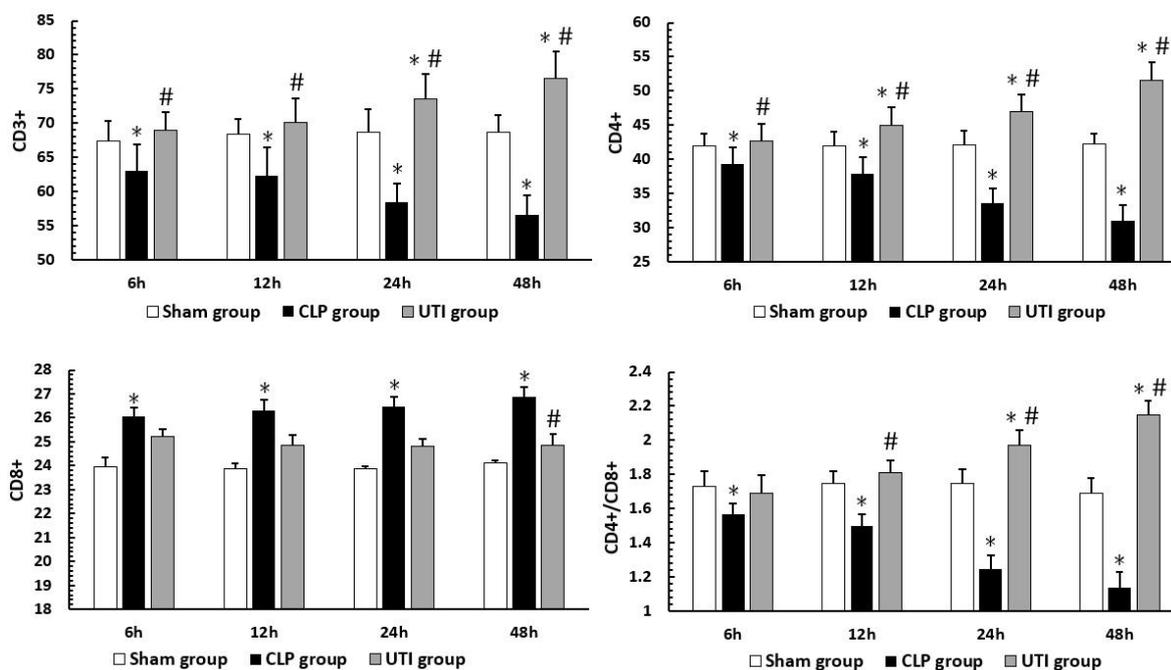


Figure 2.

T cell ratios of each group

* $p < 0.05$ compared with the Sham group; # $p < 0.05$ compared with the CLP group

TNF- α and IL-6 are early proinflammatory cytokines, involved in the early sepsis complicated by multiple-organ damage [17]. It can promote the production of free radicals, bradykinin, histamine and other substances, activate the complement and aggravate the tissue damage [18]. Th17 cells are the first effector T cells involved in anti-infective immunity [19]. The level and function size of Th17 represent the body's ability to clear pathogens [20]. The specific cytokine secreted by Th17 cells is interleukin-17 (IL-17) [21]. Chong *et al.* [22] showed that when IL-17 antibody-mediated IL-17 secretion decreases the body's ability to eliminate various pathogenic bacteria is reduced, leading to an increased bacterial burden and decreased survival rate. In this study, the levels of TNF- α and IL-6 in the CLP group increase significantly, indicating that sepsis causes the inflammatory response of the organism, which can be alleviated by the UTI treatment effectively. The plasma IL-17 concentration in the UTI group is significantly decreased compared with the CLP group, indicating that UTI treatment can reduce the high inflammatory response of the body.

CD3⁺ is expressed on the surface of all T lymphocytes, which indicates the total number of T lymphocytes in the rat organism [23]. The CD3⁺ T cells decreased immediately, 6 h after surgery in the CLP group. Although, there was no statistical significance, the downward trend suggests that there may be different degrees of immune suppression in the early stage, or that both immune suppression and hyper-function existed at the same time, so that there is no obvious change for the CD3⁺ T cells in the total number of

early sepsis, which is consistent with the pathophysiological changes of severe sepsis in the early stage. UTI treatment produces an immunity enhancement effect, CD3⁺ T cells increasing significantly 12 h after surgery. In the later period after surgery, 24 h and 48 h, there is a significant increase for CD3⁺ T cells in the UTI group compared with the CLP and Sham group.

The changes and trends of CD4⁺ T cells and CD3⁺ T cells are similar. In the early stage of sepsis, the release of a large number of inflammatory mediators consumes some CD4⁺ T cells. In the later period, the inflammatory response is controlled to a certain extent as the anti-inflammatory mediators increase, but the anti-inflammatory mediators also suppress the immune ability of the body, causing immune suppression [24]. Compared with the change of CD3⁺ T cells, the change of CD4⁺ T cells can better reflect the immune suppression of sepsis in the early stage, because it excludes the effects from changes of CD8⁺ T cells.

The main function of CD8⁺ T cells is to mediate the killing and lysis of self-cells infected by the pathogens [25]. The viral infection of virus and other factors on the cells of rats are not involved in this experiment, so there is no significant change for CD8⁺ T cells observed. It increased significantly in CLP group at 12 h, 24 h and 36 h. CD8⁺ T cells may be involved in the removal of some self-dead cells in the body, and the increase in their number may cause enhanced autoimmune cells destruction, which has a certain role in promoting the generation of the immune suppression [26]. UTI can reduce the level of CD8⁺ T cells of rats

with sepsis after CLP, reduce the destruction of the autoimmune cells, and relieve the immune suppression to some degree. CD4⁺/CD8⁺ mainly represent the immune state of the body, and it indicates the disorder of the immune state [27].

In this study, the overall mortality rate of rats in the UTI group is significantly lower than that in the other groups. Immune suppression does not occur immediately after the CLP, and the CD4⁺ and CD4⁺/CD8⁺ increased with the prolongation of the medication time ($p < 0.01$), suggesting that UTI can enhance the immunity of the organism.

Conclusions

In conclusion, UTI, as an important physiologically active substance involved in the repair of inflammatory sites and maintaining the balance of the internal environment, has a significant effect on improving the immunity. It has a definite effect on the sepsis, and can reduce the level of inflammation, improve the immunity and reduce the mortality rate.

Conflict of interest

The authors declare no conflict of interest.

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