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The impact of ulinastatin on lymphocyte apoptosis and autophagy in sepsis patients

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This study aimed to assess the influence of ulinastatin (UTI) on lymphocyte apoptosis and autophagy in sepsis patients, as well as its effect on inflammatory factors and vital organ function, with the goal of providing insights for improved clinical management of sepsis. A total of 40 sepsis patients were randomly assigned to the UTI group or the control group. The UTI group received standard treatment plus intravenous UTI, while the control group received standard treatment alone. Peripheral blood samples were collected at multiple time points for analysis of lymphocyte apoptosis, autophagy, inflammatory markers, and organ function. Various experimental techniques including Hoechst staining, transmission electron microscopy, and Western blot analysis were utilized to assess lymphocyte apoptosis, autophagy, and related protein expression levels. The study revealed that UTI treatment significantly inhibited lymphocyte apoptosis and promoted autophagy in sepsis patients. The levels of autophagy-related proteins LC3-II and Beclin-1 were substantially elevated, while the ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax was increased following UTI treatment. Furthermore, the levels of inflammatory markers IL-6, procalcitonin, and C-reactive protein were markedly reduced in the UTI group compared to the control group. Additionally, UTI treatment led to improved liver, kidney and cardiac function as evidenced by reduced levels of liver enzymes and creatinine, and cardiac enzymes. The findings of this study demonstrate that UTI exerts a protective effect on septic patients by inhibiting lymphocyte apoptosis, promoting autophagy, and attenuating systemic inflammation. Moreover, UTI treatment was associated with improved liver, kidney, and cardiac function in septic patients. These results contribute to a better understanding of the clinical management of sepsis and underscore the potential of UTI as a therapeutic intervention in septic patients.

Keywords Sepsis, Ulinastatin (UTI), Lymphocyte apoptosis, Autophagy, Inflammatory response

Sepsis is a critical condition characterized by a dysregulated response to infection, resulting in life-threatening organ dysfunction[1,2]. Common and severe in emergency departments, it affects over 19 million people annually worldwide, with a mortality rate exceeding 25%, causing more than 6 million deaths[3–5]. The imbalance of host anti-inflammatory and immune responses is the central mechanism underlying the development of life-threatening organ dysfunction in sepsis patients. It has been suggested that the reduction of lymphocytes is closely associated with the dysregulation of host anti-inflammatory and immune responses[6,7]. Lymphocytes are crucial cells in the immune system responsible for recognizing and attacking foreign invaders such as viruses and bacteria within the body. Both the quantity and quality of lymphocytes play vital roles in maintaining the balance of the immune response. Insufficient numbers of lymphocytes can lead to a decrease in the effectiveness of the immune system, making individuals more susceptible to infections. Studies have shown that lymphopenia, a decrease in lymphocyte count, is a common occurrence in individuals with sepsis and is associated with mortality[8,9]. Chung et al. found that severe lymphopenia, defined as lymphocyte counts of less than $0.5 \times 10(3)/\mu L$, was present in a significant percentage of sepsis patients upon admission[10]. Moreover, persistent lymphopenia following sepsis diagnosis has been correlated with increased mortality, underscoring the importance of lymphocytes in defending against infections[11]. Programmed cell death of lymphocytes and the role of autophagy and apoptosis in this process is of significant importance in the context of sepsis[12–14].

Autophagy is a metabolic process involving the degradation of damaged organelles and macromolecules within cytoplasmic vesicles, which fuse with lysosomes for degradation[15,16]. Under conditions such as hypoxia, nutrient deprivation, oxidative stress, microbial invasion, and stress, autophagy envelops and degrades damaged

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cell components, aging and injury, thereby generating nutrients for cellular utilization[17,18]. Thus, autophagy plays a protective role in maintaining cell survival and reducing cell apoptosis. Studies have demonstrated a close association between autophagy and the development of sepsis[13,19,20]. Lin et al. found that in sepsis, the level of T lymphocyte autophagy is significantly reduced[21]. Xu et al. also discovered that the autophagic levels of lymphocytes in septic patients are significantly suppressed[22]. Therefore, enhancing the level of lymphocyte autophagy may represent a potential strategy for the treatment of sepsis.

Ulinastatin (UTI), a glycoprotein hydrolase inhibitor derived from fresh human urine, is widely used in the treatment of diseases such as sepsis, acute pancreatitis, trauma, shock, and multiple organ dysfunction syndrome[23,24]. The use of ulinastatin in the treatment of sepsis has been shown to reduce the mortality rate and improve patient prognosis[25,26]. In this study, we evaluated the influence of ulinastatin on the autophagy and apoptosis of lymphocytes in sepsis patients, as well as its impact on inflammatory factors and organ function such as the liver, kidney and heart. Our findings aim to provide better guidance for the clinical management of sepsis.

The current understanding of lymphocyte apoptosis and autophagy in sepsis has primarily been limited to tissue rich in mitochondria, such as skeletal muscle and cardiac muscle in animal models[27,28]. this study aimed to elucidate the impact of ulinastatin on the apoptosis and autophagy of lymphocytes in sepsis patients. Additionally, we sought to observe the effects on inflammatory factors and the function of vital organs.

Materials and methods

Study population and diagnosis criteria

The study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (KY2018-257). The study was performed in accordance with the ethical standards laid out in the 1964 Declaration of Helsinki. Written informed consent was obtained from individual participants or their guardians.

The diagnosis of sepsis was based on the diagnostic criteria established by the Surviving Sepsis Campaign in 2016, with a Sequential Organ Failure Assessment (SOFA) score of at least 2 points above the baseline for infection or suspected infection[29].

Exclusion criteria

Patients with cancer, immunocompromised conditions, organ transplantation, immunosuppressive therapy, pregnancy, breastfeeding or younger than 18 years old were excluded from the study.

Experimental design

Patients meeting the inclusion criteria were randomly assigned to either the study group (UTI group) or the control group, with 20 patients in each group.

Experimental procedure

1. The control group received standard treatment, while the study group received standard treatment plus intravenous drip of 200,000 U of UTI every 12 h for 7 days.
2. Peripheral blood samples were collected from the patients at admission (day 0), 24 h after admission (day 1), day 3, and day 7 for isolation of lymphocytes and measurement of liver function (alanine aminotransferase, ALT; aspartate aminotransferase, AST), renal function (creatinine, Crea), cardiac enzymes (creatine kinase-MB, CK-MB; cardiac troponin I, cTnI), and inflammatory markers (interleukin-6, IL-6; procalcitonin, PCT; C-reactive protein, CRP). Western blot gel preparation was performed using BeyGel^{TM}SDS-PAGE pre packaged gel kit (P0057B, Beyotime), primary antibody using Anti-LC3B antibody (Ab51520, Abcam), Anti Beclin 1 antibody (EPR19662, Abcam), Bcl-2 antibody (EPR17509, Abcam), Bax antibody (E63, Abcam), GAPDH primary antibody using GAPDH antibody (Ab181602, Abcam), secondary antibody using Goal Anti Rabbit IgG (H&L) Antibody DyLight^{TM}680 Conjugated-611-144-122, Rockland Immunochemicals). IL-6 was detected using a cobas 6000 fully automated immunoassay analyzer and matching reagents (Merck); The detection of PCT uses UPT-3A upconversion luminescence immunoassay analyzer and supporting reagents (Beijing Rejing Biotechnology Co., Ltd.); The detection of CRP is carried out using the SYSMEX XN9100 blood analyzer and supporting reagents (Sysmex). The detection of ALT, AST, Crea, CK-MB, and cTnI was performed using a Dimension fully automated biochemical detector and matching reagents (Siemens).
3. The lymphocytes were separated by gradient centrifugation method and carried out under conditions of 20 ± 2 °C. Add 3 ml of human peripheral lymphocyte isolate (LTS1077, TBD) into a 15 ml centrifuge tube. Add 2 ml of blood sample onto the separation solution and centrifuge at 650g for 20 min. After centrifugation, the liquid in the centrifuge tube was divided into four layers from top to bottom, namely plasma layer, lymphocyte layer, separation liquid layer, and red blood cell layer. Use a pipette to transfer the second layer of lymphocytes into another 15 ml centrifuge tube. Add 10 ml PBS solution to the centrifuge tube, mix the cells well, and centrifuge at 250g for 10 min. Discard the supernatant. Resuspend the cells in 5 ml PBS solution and centrifuge at 250g for 10 min. Repeating steps 6 and 7, discard the supernatant, and resuspend the cells in 0.5 ml of the corresponding liquid required for the experiment.
4. Lymphocyte apoptosis was detected using Hoechst staining method. The detection of lymphocyte apoptosis was performed using the Hoechst staining kit (C0003, Beyotime).
5. Lymphocyte autophagy and apoptosis were observed using transmission electron microscopy.
6. Protein levels of LC3-II, Beclin-1, Bcl-2, and Bax in lymphocytes were detected using Western blot analysis. the detection of lymphocyte LC3-II, Beclin-1, Bcl-2, and Bax proteins was performed using the BCA protein concentration assay kit (P0010S, Beyotime).

7. The length of hospital stay was recorded for each patient.

Detection of lymphocyte apoptosis by hoechst staining

Isolated lymphocytes were fixed with 4% paraformaldehyde for 15 min and then incubated with Hoechst 33,258 dye for 10 min at room temperature. After washing with phosphate-buffered saline (PBS), the stained cells were observed under a fluorescence microscope (excitation wavelength 350 nm, emission wavelength 461 nm) to detect apoptotic cells characterized by condensed and fragmented nuclei.

Electron microscopy for observation of lymphocyte autophagy and apoptosis

Isolated lymphocytes were fixed with 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in a series of ethanol solutions, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope to visualize autophagosomes, autolysosomes, and apoptotic bodies.

Western blot analysis for detection of autophagy and apoptosis-related proteins

Cellular proteins were extracted from lymphocytes using RIPA lysis buffer, and the concentration was determined using a BCA protein assay kit. Equal amounts of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with specific antibodies against LC3-II, Beclin-1, Bcl-2, and Bax, followed by incubation with HRP-conjugated secondary antibodies. Protein bands were visualized using an enhanced chemiluminescence detection system, and densitometry analysis was performed using ImageJ software.

Statistical analysis

All the data collected in this study were analyzed using SPSS 24.0 software and GraphPad Prism 5 software. Normally distributed measurement data were expressed as mean ± standard deviation (SD), and the comparisons were examined by Student-t test. $p < 0.05$ was considered statistically significant.

Results

UTI inhibits lymphocyte apoptosis

Using the Hoechst staining method to detect lymphocyte apoptosis, it was observed that the apoptosis level in the control group gradually increased from day 0 to day 1 and day 3, with a slight decrease at day 7 but still higher than at day 0. In comparison to the control group, the study group showed significantly reduced apoptosis levels at each time point (Fig. 1), suggesting that UTI inhibits lymphocyte apoptosis levels.

UTI promotes autophagy in lymphocytes

Transmission electron microscopy revealed that in the study group, on day 0, lymphocytes exhibited swelling, organelle disappearance, and incomplete cell membranes, with observable autophagosomes and apoptotic bodies. On day 1, the number of autophagosomes in lymphocytes increased compared to day 0. By day 3, lymphocytes displayed the highest number of autophagosomes, with no signs of apoptosis. On day 7, although the level of autophagy decreased compared to day 3, autophagosomes were still observable. Compared to the control group, the study group showed reduced apoptosis and increased autophagy (Fig. 2).

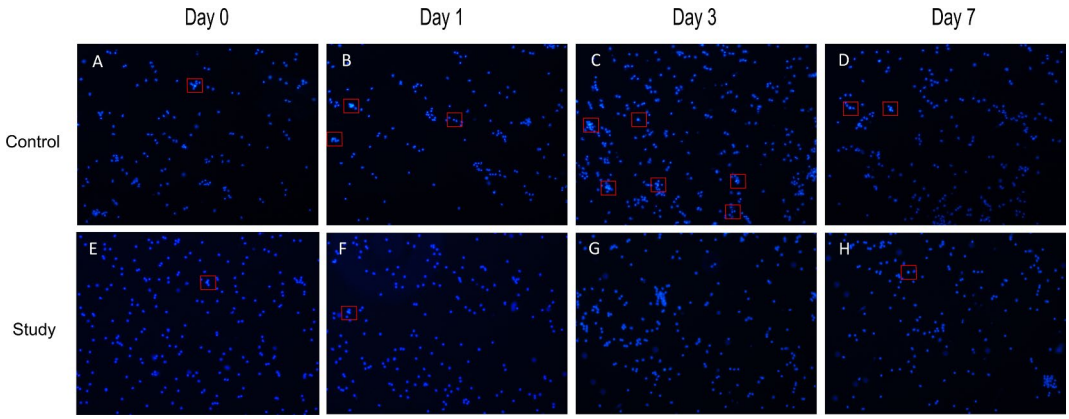

Fig. 1. Hoechst staining revealed a decrease in lymphocyte apoptosis in the study group. The cells indicated by the red border are apoptotic cells. (**A**) Apoptosis of lymphocytes was observed, which showed cell size reduction, wrinkled, nucleus shrinkage and dense staining; (**B**) The apoptotic lymphocytes increased from day 0; (**C**) The apoptotic lymphocytes were significantly higher than those on day 1, and were at the highest level in the experimental observation period; (**D**) The apoptotic lymphocytes were lower than that on day 3, but still higher than that on day 0; (**E**) Apoptosis of lymphocytes was observed, which showed cell size reduction, wrinkled, nucleus shrinkage and dense staining; (**F**) Apoptotic lymphocytes decreased from day 0; (**G**) The apoptotic lymphocytes were at the lowest level, with almost no apoptotic lymphocytes; (**H**) Although apoptotic lymphocytes were higher than those on day 3, they were still at a low level.

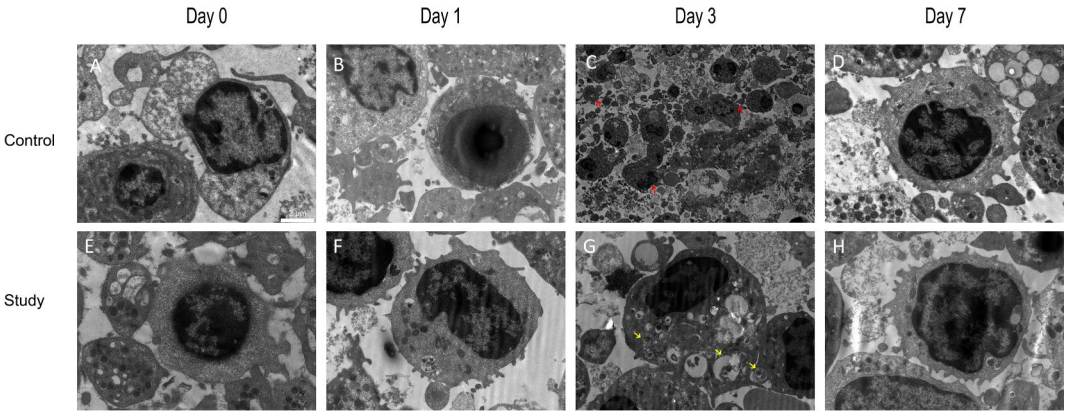

Fig. 2. Images of autophagy and apoptosis under transmission electron microscopy. The red arrow indicates cell apoptosis, while the yellow arrow indicates autophagy. (**A**) Lymphocyte swelling, disappearance of organelles, incomplete cell membrane, autophagosomes and apoptotic bodies can be observed; (**B**) The number of lymphocyte autophagosomes decreased compared with day 0, and cell apoptosis increased; (**C**) The lymphocyte has the least autophagosomes, almost no autophagosomes, and the apoptosis or necrosis of cells is the most serious; (**D**) There were more autophagosomes than on day 3, but the level was still low; (**E**) Lymphocyte swelling, disappearance of organelles, incomplete cell membrane, autophagosomes and apoptotic bodies can be observed; (**F**) The number of lymphocyte autophagosomes increased compared with day 0; (**G**) The lymphocytes had the most autophagosomes and no signs of apoptosis; (**H**) Although the level of autophagy was lower than that on day 3, autophagosomes were still observed.

UTI promotes autophagy in lymphocytes and inhibits lymphocyte apoptosis

To further confirm the changes in apoptosis and autophagy levels in the study group, we examined the protein levels of lymphocyte LC3II, Beclin-1, Bcl-2, and Bax. We found that compared to the control group, the levels of autophagy-related proteins LC3II and Beclin-1 were significantly increased in the study group. The anti-apoptotic protein Bcl-2 significantly increased after UTI treatment, while the pro-apoptotic protein Bax levels significantly decreased following UTI treatment (Fig. 3). These results indicate that UTI treatment promotes autophagy and inhibits apoptosis.

UTI treatment suppresses cellular inflammation

On the other hand, we observed the levels of inflammation-related markers IL-6, PCT, and CRP. In comparison to the control group, although we did not observe significant reductions in IL-6, PCT, and CRP in the study group at day 1 and day 3 ($p > 0.05$), while we found that on day 7, compared to the control group, the levels of IL-6, PCT, and CRP were significantly reduced in the study group ($p < 0.05$) (Fig. 4).

UTI treatment improves hepatic, renal and cardiac function

Additionally, we assessed the differences in liver function markers ALT and AST levels between the two groups, as well as kidney function (Crea), and cardiac enzymes (CK-MB, cTnI). We found that compared to the control group, the study group exhibited significantly reduced levels of ALT, AST, Crea, CK-MB, and cTnI (Fig. 5). This suggests that UTI treatment improves liver, kidney and cardiac function in septic patients.

Length of hospital stay

The duration of hospitalization can reflect treatment efficacy. We observed that the study group had a hospital stay of 9.95 ± 1.19 days, while the control group had a hospital stay of 11.85 ± 1.93 days. The hospital stay was significantly shorter in the study group compared to the control group ($p < 0.001$).

Discussion

In this study, we investigated the effects of UTI on lymphocyte apoptosis and autophagy in septic patients, as well as its impact on inflammatory factors and the function of organs such as the liver, kidneys and heart. We found that UTI has a significant protective effect in septic patients.

Research suggests that excessive lymphocyte apoptosis in septic patients can lead to immune dysfunction, multiple organ failure, increased mortality, and reducing lymphocyte apoptosis can significantly improve the prognosis of septic patients[30,31]. Preventing and reducing lymphocyte apoptosis has become an important aspect of treating sepsis[32–34]. In this study, we observed a significant reduction in lymphocyte apoptosis in septic patients receiving UTI treatment. The decrease in lymphocyte apoptosis rate in patients treated with UTI may be attributed to its impact on cellular autophagy. We found that UTI can promote lymphocyte autophagy in septic patients, thereby reducing apoptosis.

During sepsis, systemic inflammatory responses and cellular damage can activate autophagic responses to counteract the damaging effects of various external factors on cells. For instance, Krakauer et al. found that rapamycin-induced autophagy helped mice resist staphylococcal enterotoxin-induced septic shock and cardiac

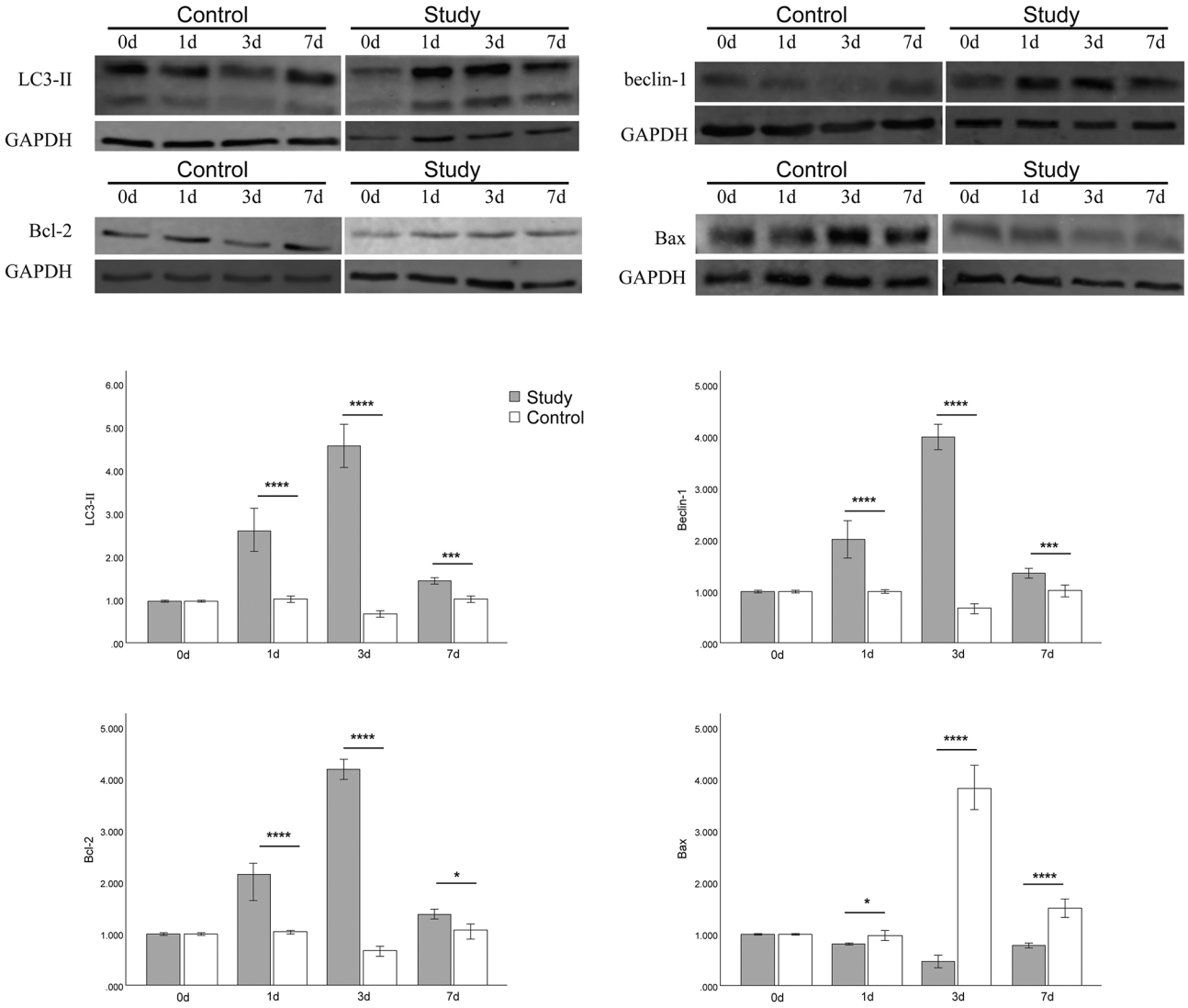

Fig. 3. Immunoblotting images of autophagy and apoptosis proteins LC3II, Beclin-1, Bcl-2 and Bax alongside corresponding statistical charts. The bar chart displays the statistical results of the expression levels of relevant proteins in the control group and the study group at different time points (Day 0, Day 1, Day 3, and Day 7). *indicates $p < 0.05$ between the control and study groups; ***indicates $p < 0.001$ between the control and study groups; ****indicates $p < 0.0001$ between the control and study groups.

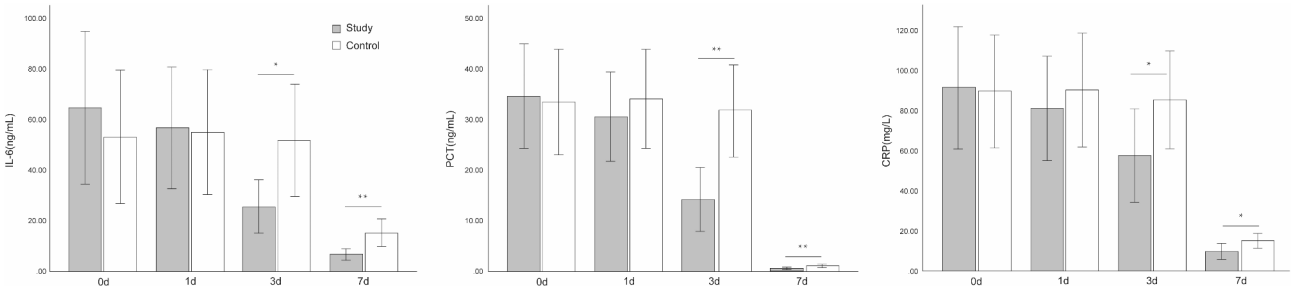

Fig. 4. Statistical chart of inflammatory factors IL-6, PCT, and CRP. The bar chart displays the statistical results of inflammatory factors in the control group and the study group at different time points (Day 0, Day 1, Day 3, and Day 7). *indicates $p < 0.05$ between the control and study groups; **indicates $p < 0.01$ between the control and study groups.

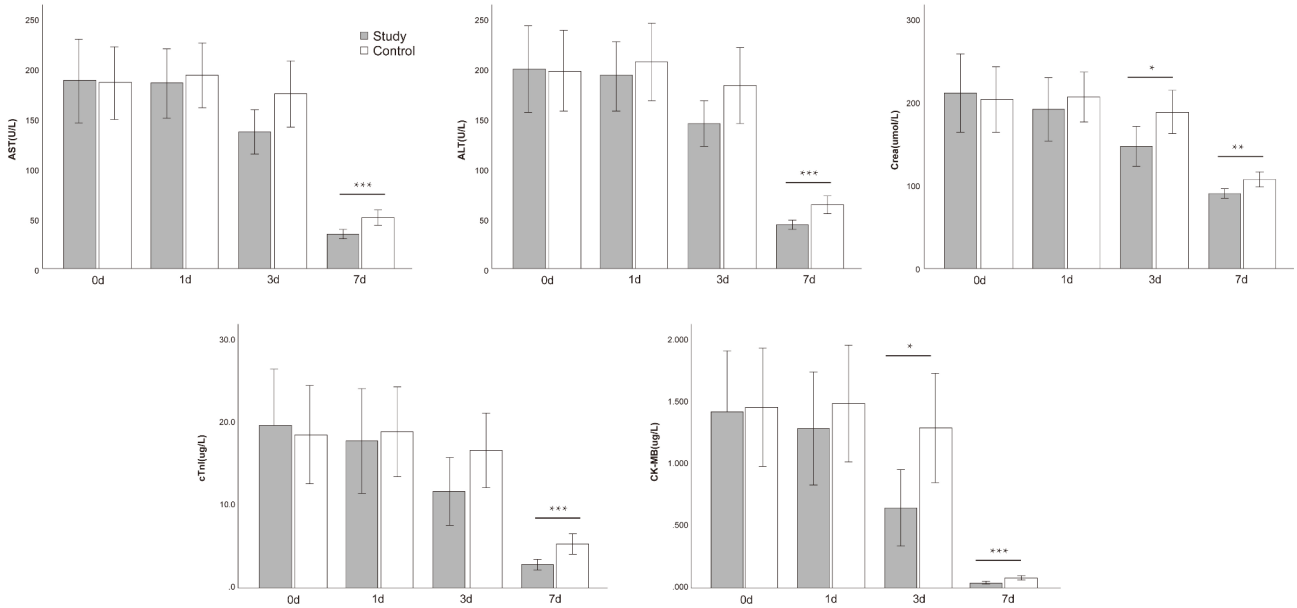

Fig. 5. Statistical chart of liver, kidney and cardiac function indicators: ALT, AST, Crea, CK-MB, and cTnI. The bar chart displays the statistical results of function indicators in the control group and the study group at different time points (Day 0, Day 1, Day 3, and Day 7). *indicates $p < 0.05$ between the control and study groups; **indicates $p < 0.01$ between the control and study groups; ***indicates $p < 0.001$ between the control and study groups.

dysfunction[33]; Sun et al. found that p53 deacetylation alleviated acute kidney injury caused by sepsis by promoting autophagy[35]; Takahashi et al. found that the inhibition of autophagy with chloroquine significantly increased mortality in septicemia[36]. By measuring the levels of autophagy-related proteins LC3-II and Beclin-1, we observed elevated expression levels of these two autophagic markers in the UTI treatment group, further confirming UTI's promotive effect on lymphocyte autophagy. This suggests that UTI significantly influences apoptosis occurrence in septic patients by modulating the process of cellular autophagy.

Systemic inflammatory response plays a crucial role in the occurrence and progression of sepsis, being a significant cause of multiple organ dysfunction[37]. Inflammatory markers such as IL-6, CRP, and PCT are markedly elevated during sepsis[38,39]. Particularly, IL-6, produced by various cell types including B cells, T cells, and macrophages, possesses multiple regulatory functions such as cell proliferation, differentiation, acute-phase response, and immune response. IL-6 occupies a central position in the cascade of inflammatory reactions, initiating and amplifying inflammatory responses, participating in the entire process of inflammation, and affecting the functions of multiple organs in the body through the inflammation, immune, and hematopoietic systems[40,41]. Therefore, effectively suppressing the body's inflammatory response is key to the successful early treatment of septic patients. Studies have shown that lymphocyte autophagy activity is strong and can inhibit lymphocyte apoptosis, which is beneficial for controlling inflammatory responses and achieving a balance between pro-inflammatory and anti-inflammatory processes[37,42]. We observed that UTI treatment significantly reduces the levels of inflammatory factors in septic patients. IL-6, PCT, and CRP, as inflammatory markers, all showed significant decreases in the UTI treatment group, indicating that UTI can effectively suppress the occurrence of inflammatory responses. This is closely related to the effect of UTI on lymphocytes, and the lower levels of inflammatory factors may be due to the reduction in lymphocyte apoptosis-induced immune dysfunction, thereby alleviating the inflammatory response. Therefore, UTI may affect the levels of inflammatory factors in septic patients by modulating lymphocyte apoptosis and related autophagic processes.

Additionally, we observed that UTI treatment exerts a protective effect on organ function, including the liver, kidneys, and heart, in septic patients. This observation is closely related to the effects of UTI on lymphocyte apoptosis and autophagy. By reducing the rate of lymphocyte apoptosis, UTI may provide protection for patients' immune function, thereby alleviating organ damage caused by systemic inflammatory responses.

Despite the significant findings of this study, several limitations should be acknowledged. Firstly, the sample size of the study was relatively small, which may have restricted the generalizability of the results. A larger cohort of sepsis patients could provide more robust evidence for the effects of ulinastatin on lymphocyte apoptosis, autophagy, and inflammatory markers. Additionally, this study focused on the short-term effects of ulinastatin treatment over a period of 7 days. Long-term effects and potential complications of ulinastatin therapy were not evaluated in this study. Future research investigating the extended influence of ulinastatin on lymphocyte function and patient outcomes is warranted. Moreover, while this study assessed the impact of ulinastatin on lymphocyte apoptosis and autophagy, the specific underlying molecular mechanisms mediating these effects were not fully elucidated. Further investigations into the signaling pathways involved in ulinastatin-mediated

modulation of lymphocyte function are necessary to provide a comprehensive understanding of its therapeutic actions in sepsis.

In summary, the results of this study indicate that UTI improves the immune function of septic patients by promoting lymphocyte autophagy and reducing the rate of cell apoptosis, consequently alleviating inflammatory responses and multi-organ damage. Therefore, UTI may be exerting significant protective effects on septic patients by regulating the process of lymphocyte autophagy. However, further clinical research is needed to validate the therapeutic efficacy of UTI in septic patients and elucidate its underlying mechanisms.

Data availability

All data are available in the main text or the supplementary materials.

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Author contributions
DZ and YD designed the study. DZ, JS, JZ, YW and JD performed the experiments and acquired the data. DZ, JS and JZ analyzed the data. DZ, YW and JD prepared the figures. YD supervised the study. DZ, JS and YD drafter the original manuscript. All authors contributed to manuscript editing and writing. All authors read and approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
The study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (KY2018-257). The study was performed in accordance with the ethical standards as laid out in the 1964 Declaration of Helsinki. Written informed consent was obtained from individual participants or their guardian.

Additional information
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