Abstract. – OBJECTIVE: By constructing the severe burns model in rat, we explored the effects of different doses of Ulinastatin (UTI) on protecting myocardium from oxidative stress and inflammatory reaction.

MATERIALS AND METHODS: The severe burns model in rat was first constructed. Burned rats were intervened with different doses of UTI. Contents of cardiac troponin I (cTnI), Interleukin-1 (IL-1), Interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in rat serum and heart homogenate were detected by enzyme-linked immunosorbent assay (ELISA). Activities of SOD (superoxide dismutase), CAT (catalase), GSH-Px (glutathione peroxidase), and MDA (malondialdehyde) were detected by commercial kits. The inflammation and pathological changes in rat heart were observed by HE (Hematoxylin-Eosin) staining. Protein expressions of Cox-2, iNOS, NF-κB, Nrf2, and HO-1 in rat myocardium were detected by Western blot.

RESULTS: Higher levels of cTnI, IL-1, IL-6, and TNF-α were found in model group than those of control group \((p<0.05)\). Besides, decreased contents of cTnI, IL-1, IL-6, and TNF-α were observed in both UTI 50 ku/kg group and UTI 100 ku/kg group compared with those of model group \((p<0.05)\). Decreased activities of SOD, CAT, and GSH-Px, as well as increased MDA level in heart were observed by HE (Hematoxylin-Eosin) staining. Protein expressions of Cox-2, iNOS, NF-κB, Nrf2, and HO-1 in rat myocardium were detected by Western blot.

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CONCLUSIONS: Ulinastatin alleviates myocardial injury induced by severe burns. It exerts a protective role in myocardium via inhibiting oxidative stress and inflammatory response.

Key Words: Severe burns, Myocardial injury, Oxidative stress, Inflammatory response, Ulinastatin.

Introduction

Burns are tissue and organ damages caused by boiling water, hot oil, intense light, fire, electricity, radiation or strong alkaline chemicals on the body\(^1,2\). Among them, thermal burns are the most common type, accounting for 85% to 90% of the total burns cases\(^3\). Severe burns are 2nd-degree burns over 30% of the total body surface and 3rd-degree burns over 10% of the total body surface, which are the main types of body damage and death\(^4\). Severe burns easily lead to fluid exudation, infections, impaired distal organ ischemia, and high metabolic syndrome. More seriously, high metabolic syndrome continues to exist after burns, thus resulting in the glucose metabolism disorder, short-term malnutrition, decreased immunity, severe infection, and even organ failure\(^5\). Severe burns result in systemic inflammatory responses and multiple organ dysfunction syndrome with high mortality\(^6,7\).
Burned patients in the middle and early stages often suffer from shock, which greatly affects the organ function and infection control. The pathogenesis of burn shock is very complicated and has not yet been fully understood. Among them, blood volume decline and tissue perfusion caused by increased capillary permeability after burns are recognized as the important causes of burn shock. With the in-depth researches, it has been found that the occurrence of burn shock is not only related to blood volume decline, but also closely related to vascular reactivity and cardiac pump function. Insufficient blood perfusion and impaired circulatory system function remarkably aggravate clinical outcomes of burned patients.

Studies have shown that in the early stage of burns, myocardial damage and heart function decline are the first symptoms prior to other organ damages, which are second only to lung injury. Besides, myocardial tissue has already undergone hypoxic-ischemic damage before blood volume decline. Therefore, the early occurrence of myocardial damage is considered the initial factor that induces or aggravates burn shock. After severe burns, the degree of myocardial damage is significantly associated with cardiac function decline, which may further affect functions of other major organs.

Ulinastatin (UTI) has been applied in treating acute pancreatitis since the mid 1980s. It is reported that UTI can reduce the serum level of hsCRP (high-sensitive acute-phase reaction protein C) and APACHE II (Acute Physiology And Chronic Health Evaluation II) score in critical surgical patients. UTI is a protease inhibitor with a broad spectrum. Relative studies have confirmed that UTI can scavenge oxygen radicals produced by myocardial ischemia and reperfusion, thereafter improving myocardial reperfusion injury via reducing cardiomyocyte apoptosis.

In our study, we explored the effect of UTI on inflammatory response and oxidative stress in severely burned rats. We aim to investigate the specific role of UTI in protecting myocardial tissues, which provides a theoretical basis for clinical burned patients.

**Materials and Methods**

**Experimental Animals**

40 Wistar rats were adaptively fed for 1 week and randomly assigned into control group, model group, UTI 50 ku/kg group, and UTI 100 ku/kg group, with 10 rats in each group. Rats in control group did not receive any procedures and were sacrificed one week later. Rats in model group underwent thermal burn and sacrificed one week later. Rats in UTI 50 ku/kg group and UTI 100 ku/kg group received an intraperitoneal injection of 50 ku/kg or 100 ku/kg UTI after thermal burn, respectively. This study was approved by the Animal Ethics Committee of Nanjing Medical University Animal Center, China.

**Construction of Severe Burns Model in Rat**

The construction of severe burns model in rat was modified based on the previously described method. 30% of total body surface area of 3\(^{rd}\)-degree burns was calculated using the formula: \( A (\text{cm}^2) = K \times W^{2/3} \) (A: body surface of rat; K = 9.1; W: body weight). Briefly, rats’ back were shaved with 8% sodium sulfide and washed with warm water. Rats were anesthetized with intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g). After labeling the lesion area, the 3\(^{rd}\)-degree burns was induced by thermal burn of 97±1°C water for 18 s. Intraperitoneal injection of 4 mL/100 g saline was immediately administered for preventing shock.

**Sample Collection**

After anesthesia, 4-5 mL of blood samples were collected from rat abdominal aorta and preserved at 4°C for 2 h. Blood was then centrifuged at 4000 rpm/min for 15 min at room temperature, and 200 µL of supernatant was placed in EP tube for preservation at -80°C. Serum levels of Ang II, cardiac troponin I (cTnI), Interleukin-1 (IL-1), Interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were detected.

**Oxidative Stress Indicator Detection**

Heart tissues were collected, washed with pre-cooled saline and fixed in 4% paraformaldehyde. Part of rat heart was then paraffin embedded and sliced for immunohistochemistry. The remaining part of heart tissue was preserved in liquid nitrogen for homogenate preparation. 300 mg of heart tissue was homogenated in 1 ml of pre-cooled saline for 5 min, centrifuged at 3000 g/min for 15 min at 4°C, and preserved at -80°C. Contents of SOD (superoxide dismutase), CAT (catalase), GSH-Px (glutathione peroxidase), and MDA (malondialdehyde) in heart homogenate were further detected.
HE (Hematoxylin-Eosin) Staining
Heart tissues were fixed in 4% paraformaldehyde for 48 h, followed by gradient dehydration with ethanol. Subsequently, heart tissues were paraffin embedded and sliced for HE staining (Boster, Wuhan, China).

ELISA (Enzyme-Linked Immunosorbent Assay)
 Serum samples were centrifuged at 3000 g/min for 10 min. The supernatant was collected for ELISA detection. Contents of Ang II, cTnI, IL-1, IL-6, and TNF-α were detected according to the instructions of ELISA kit (Abcam, Cambridge, MA, USA). The optical density (OD) at the wavelength of 450 nm was detected using a microplate reader (Bio-Rad, Hercules, CA, USA).

Detection of SOD, CAT, GSH-Px and MDA Activities in Heart Homogenate
Heart homogenate was centrifuged at 3000 g/min for 10 min. The supernatant was collected for detecting SOD (superoxide dismutase), CAT (catalase), GSH-Px (glutathione peroxidase), and MDA (malondialdehyde) activities by relative commercial kits (Jiancheng, Nanjing, China).

Western Blot
Protein samples were separated by 10% polyacrylamide gel electrophoresis. After transferred to PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA), the protein-contained membrane was blocked with 5% skim milk for 2 h. The corresponding primary antibody was added and incubated with proteins at 4°C overnight, followed by incubation of secondary antibody for another 1 h. The ECL (enhanced chemiluminescence) luminescent agent (Thermo Fisher Scientific, Waltham, MA, USA) was used to develop the protein imprint. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal reference here.

Statistical Analysis
Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) was used for data processing and analysis. The data were expressed as mean±SD. The t-test was used to compare the mean values of two independent samples. One-way ANOVA was used for comparing differences among groups, followed by Post-Hoc Test (Least Significant Difference). p<0.05 was considered statistically significant.

Results

Contents of cTnI, IL-1, IL-6, and TNF-α in Rat Serum and Heart Homogenate
ELISA data suggested higher levels of cTnI, IL-1, IL-6, and TNF-α in model group than those of control group (p<0.05, Figure 1). Besides, decreased contents of cTnI, IL-1, IL-6, and TNF-α were observed in both UTI 50 ku/kg group and UTI 100 ku/kg group compared with those of model group (p<0.05, Figure 2).

Activities of SOD, CAT, GSH-Px, and MDA in Rat Heart Homogenate
Decreased activities of SOD, CAT, and GSH-Px were observed in model group than those of control group (p<0.05). However, UTI treatment remarkably elevated SOD, CAT, and GSH-Px activities in burned rats (p<0.05, Figure 3A-3C). On the contrary, MDA level was elevated in model group than that of control group (p<0.05). Decreased MDA level was found in both UTI 50 ku/kg group and UTI 100 ku/kg group compared with those of model group (p<0.05, Figure 3D).

Pathological Changes of Rat Myocardium
We did not observe a remarkable pathological change of rat myocardium in the control group (Figure 4A). However, abundant infiltration of inflammatory cells was found in rat myocardium of model group (Figure 4B). In UTI 50 ku/kg group, the inflammation infiltration became milder than that of model group (Figure 4C). UTI 100 ku/kg exhibited the mildest inflammatory response in the myocardium (Figure 4D).

Protein Expressions of Inflammation and Oxidative Stress-Related Genes in Myocardium
Upregulated protein expressions of Cox-2, iNOS, and NF-κB were found in model group compared with those of control group (p<0.05, Figure 5A), which were remarkably decreased in UTI group (p<0.05, Figure 5B). Besides, protein expressions of Nrf2 and HO-1 were downregulated in model group than those of control group (p<0.05, Figure 5C), which were upregulated in UTI group (p<0.05, Figure 5D).

Discussion
Burns are complex traumatic diseases caused by heat, chemical substances, electric currents,
Accumulated evidence has shown that severe burns not only causes skin and deep tissue damage, but also damages various internal organs. Organ dysfunction further influences the progression and prognosis of burns. Early damages of lung, heart, kidney, and digestive tracts are observed after severe burns. Among them, myocardium injury after severe burns leads to weakened pump function and effective circulating blood volume reduction, which seriously affect the clinical outcomes of burned patients.\(^{20-22}\) Therefore, it is particularly crucial to study the mechanism of post-burn myocardial injury and the protection approaches.

Animal models are of great significance in exploring the pathogenesis and treatment of severe burns. In this study, rats underwent thermal burns under 97±1°C hot water after labeling the burned area on the back. The procedure is simple with accurate burned depth and area, which is an ideal method for constructing burned animal model.

Loss of large amounts of fluid after severe burns directly causes myocardial ischemic and hypoxic damage. In addition, stress response, uncontrolled inflammatory response, ischemia-reperfusion injury after severe burns all lead to the structure and function alterations in

**Figure 1.** Serum levels of inflammatory factors in rats. **A,** Serum level of cTnl in the four groups. **B,** Serum level of IL-1 in the four groups. **C,** Serum level of IL-6 in the four groups. **D,** Serum level of TNF-α in the four groups. *p*<0.05: Compared with control group; #*p*<0.05: Compared with model group; &*p*<0.05: Compared with UTI group.
vascular endothelial cells and neutrophils. These alterations further result in microcirculatory disturbance, myocardial ischemia, and hypoxia injury. Although the fluid administration and blood volume supply are effectively performed, myocardial ischemic and hypoxic damage cannot be avoided. Increased vascular permeability and decreased effective circulating blood volume after burns further lead to low perfusion and hypoxia in various organ tissues. However, reperfusion of tissues and organs may develop into ischemia/reperfusion injury. Studies have shown that ischemia/reperfusion produces a large amount of inflammatory mediators, followed by inflammatory chain reaction and MODS (multiple organ dysfunction syndrome). Meanwhile, the oxidative stress reduced by inflammation further damages myocardium. Among multiple inflammatory mediators, IL-1 has a negative inotropic effect on the myocardium and inhibits myocardial contractility. In our study, levels of cTnI, IL-1, IL-6, and TNF-α in serum and heart homogenate of burned rats were remarkably elevated, indicating inflammatory response after severe burns. UTI treatment markedly downregulated these inflammatory mediators, especially in UTI 100 ku/kg group.

**Figure 2.** Levels of inflammatory factors in rat myocardium. A. cTnI level in rat myocardium of the four groups. B. IL-1 level in rat myocardium of the four groups. C. IL-6 level in rat myocardium of the four groups. D. TNF-α level in rat myocardium of the four groups. *p<0.05: Compared with control group; #p<0.05: Compared with model group; &p<0.05: Compared with UTI group.
Under Normal Circumstances, the Body has a Complete Antioxidant Defense System

The dynamic balance of generation and elimination of oxygen free radicals prevents oxygen radical reactions and blocks the lipid peroxidation chain reaction. A small amount of reactive oxygen species regulates the normal physiological functions of cardiomyocytes. However, abundant oxygen free radicals are produced after burns\textsuperscript{31}. ROS accumulation results in lipid peroxidation, ATP consumption, energy metabolism disorders, and electrolyte disturbance\textsuperscript{32}. Relative studies have found that oxygen free radicals are generated in large quantities after ischemia-reperfusion injury\textsuperscript{33}. In our work, levels of SOD, CAT, and GSH-Px were remarkably decreased in burned rats compared with those of controls. Meanwhile, MDA level in the myocardium was elevated, indicating the increased lipid peroxidation after severe burns. On the contrary, UTI treatment remarkably alleviated these changes, suggesting its potential role in protecting myocardial function induced by severe burns.

Cyclooxygenase (Cox) is a rate-limiting enzyme that catalyzes the synthesis of thromboxanes...
A2 (TXA2) and prostaglandin (PG) by arachidonic acid (AA). Under physiological conditions, Cox-2 is expressed in extremely low copy number in most tissues. However, Cox-2 can be activated by lipopolysaccharide, TNF, IL-1, epidermal growth factor, and platelet activating factor. Cox-2 exerts an essential role in the inflammatory response, which is one of the important determinants of inflammatory response-mediated cytotoxicity. Some studies have shown that the Keap1-Nrf2/ARE pathway is a vital pathway that defends oxidative stress. Nrf2 is a crucial transcription factor that protects the cell against oxygen free radicals. Functionally, Nrf2 activation induces multiple antioxidants and detoxifying enzymes, accelerates the enzymatic reaction and maintains intracellular redox level. Our data showed that expression levels of Cox-2, iNOS, and NF-κB were upregulated in myocardial tissues of burned rats. Besides, expression levels of Nrf2 and HO-1 were downregulated, indicating the elevated oxidative stress and inflammatory response in burned rats. In UTI group, these pathological changes were reversed by UTI pretreatment in a dose-dependent manner.

To sum up, severe burns results in myocardial injury, inflammatory response, and lipid peroxidation. However, UTI pretreatment could alleviate the above-mentioned pathological changes via downregulating Cox-2 and iNOS, as well as upregulating Nrf2 and HO-1.

Conclusions

We showed that ulinastatin alleviates myocardial injury induced by severe burns. It exerts a protective role in myocardium via inhibiting oxidative stress and inflammatory response.
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**Conflict of Interest**
The Authors declare that they have no conflict of interest.

**References**


Effects of Ulinastatin on myocardial oxidative stress and inflammation in severely burned rats


