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Ulinastatin improves myocardial ischemia-reperfusion injury in rats through endoplasmic reticulum stress-induced apoptosis pathway

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Abstract. – OBJECTIVE: To investigate the protective role of ulinastatin (UTI) on myocardial ischemia-reperfusion (I/R) injury in rats via endoplasmic reticulum stress (ERS)-induced apoptosis pathway.

MATERIALS AND METHODS: A total of 60 rats were randomly divided into normal group (n=20), myocardial I/R model group (model group, n=20), and myocardial I/R model+UTI treatment group (treatment group, n=20). The myocardial function indicators [creatinine (Scr) and creatine kinase (CK)] were detected. Enzyme-linked immunosorbent assay (ELISA) was performed to measure serum levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and matrix metalloproteinase-9 (MMP-9). Meanwhile, the contents of reactive oxygen species (ROS), superoxide dismutase (SOD), and malondialdehyde (MDA) in rat left ventricular tissues were determined by ELISA as well. The cardiac function indexes were determined via magnetic resonance imaging (MRI) and echocardiography (ECG). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining assay was carried out to detect the apoptosis of myocardial tissues. Additionally, the expression levels of endoplasmic reticulum stress and apoptosis genes were measured through quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) assay and Western blotting analysis, respectively.

RESULTS: Serum levels of alanine aminotransferase (ALT), CK, and Scr in model group were significantly higher than those in normal group ($p<0.05$). Besides, rats in model group had significantly lowered SOD, ejection fraction (EF, %), and fractional shortening (FS, %) than those in normal group ($p<0.05$). In addition, remarkably increased contents of TNF-α, IL-6, MMP-9, MDA, and ROS, as well as higher left ventricular end-diastolic diameter (LVEDd) and left ventricular end-systolic diameter (LVESd) were observed in model group in comparison with normal group ($p<0.05$). TUNEL staining results revealed that there were more apoptotic cells in model group than that in the other two groups ($p<0.05$). Expression levels of cysteine aspartic acid-specific protease 12 (Caspase-12) and glucose-regulated protein 78 (GRP78) were evidently higher in model group than those in normal group ($p<0.05$), while the expression level of B-cell lymphoma 2 (Bcl-2) was clearly lower in model group than that in normal group ($p<0.05$). UTI treatment partially reversed the above expression changes ($p<0.05$).

CONCLUSIONS: UTI has a protective effect against myocardial I/R injury in rats by repressing the occurrence of ERS-induced apoptosis.

Key Words:
Ulinastatin, Endoplasmic reticulum stress, Apoptosis, Myocardial ischemia-reperfusion injury, Rat.

Introduction

Myocardial ischemia-reperfusion (I/R) injury, a kind of myocardial injury caused by recovery of coronary blood flow after ischemia[1], may occur in the removal of aortic cross-clamps during cardiac surgeries or primary coronary intervention. I/R leads to severe arrhythmia, endothelium dysfunction, myocardial coma and cell necrosis, apoptosis, and death, with a relatively high mortality rate[2]. Once myocardial ischemia occurs, focal cells in the ischemic area are rapidly necrotic and dead following severe blood flow occlusion. Damaged cells in the peripheral blood zone with moderate blood circulation may be survived and

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saved through prompt intervention[3]. At present, mechanisms underlying myocardial ischemia and myocardial I/R injury have been well explored during the past several decades. Myocardial ischemia leads to characteristic changes in metabolism and ultrastructure, resulting in irreversible injury. Myocardial I/R is preferred for the treatment of myocardial infarction; however, it may lead to cell death[4]. Several drugs and mechanical strategies (such as ischemic pre-conditioning or post-conditioning) have been developed to repress myocardial I/R injury, but no existing drugs are particularly effective to prevent myocardial I/R injury due to its complex multi-factor mechanisms[5,6]. Myocardial I/R injury involves reactive oxygen species (ROS) production, pro-inflammatory cytokine activation, apoptosis, neutrophil infiltration, and endothelial cell dysfunction[7]. Oxidative stress inhibition and apoptosis may be new potential strategies for myocardial ischemic diseases. Therefore, suppressing cell stress and apoptosis may contribute to relieve myocardial I/R injury.

Excessive endoplasmic reticulum stress (ERS) is one of the important pathological mechanisms of I/R injury[8]. Unfolded or misfolded proteins are accumulated in the ER lumen, which is known as ERS[9,10]. Excessive and prolonged ERS is capable of activating apoptosis through the CCAAT/enhancer-binding protein homologous protein (CHOP), Cysteine aspartic acid-specific protease 12 (Caspase-12) and other signaling pathways, ultimately resulting in cell death[11]. Animal studies[12] have pointed out that ERS-induced apoptosis due to oxidative stress plays a vital role in I/R injury. On the one hand, the production of ROS is considered as the leading cause of reperfusion injury. Besides, excessive production of ROS promotes oxidative stress-induced apoptosis by altering the intracellular antioxidant defense system[13]. On the other hand, ERS is a key event in I/R injury[14]. Reperfusion injury leads to aggravated accumulation of unfolded proteins, thus causing persistent unfolded protein response (UPR) and ultimately resulting in apoptosis. Targeting ERS has an obvious protective effect against I/R injury[15,16]. Ulinastatin (UTI), a protease inhibitor extracted from human urine, has been demonstrated to protect cerebral I/R injury[17]. Abu-Amara et al[18] stated that the protective effect of UTI relies on its anti-inflammatory and anti-oxidative stress responses. Given this, repressing ERS-induced apoptosis may be promising for the treatment of I/R injury. However, the effect of UTI on ERS-induced apoptosis during myocardial I/R needs to be further explored.

In this research, the expressions of cardiac function indexes and ERS- and apoptosis-related proteins in myocardial I/R rats were detected. The protective effect of UTI on myocardial I/R injury in rats through ERS-induced apoptosis was observed to provide important experimental support and theoretical bases for UTI treatment of myocardial I/R injury.

Materials and Methods

Instruments and Reagents

Sprague-Dawley (SD) rats (Shanghai Institutes for Biological Sciences, Shanghai, China); UTI (Tianpu Pharmaceutical Co., Ltd., Tianpu, China); enzyme-linked immunosorbent assay (ELISA) kits for interleukin-1 (IL-1), IL-6, malondialdehyde (MDA), ROS, etc. (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China); pentobarbital (Sigma, Louis, MO, USA); radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Beijing, China); β-actin, secondary antibody and primary antibody (CST, Danvers, MA, USA); microsyringe (WPI), tissue homogenizer (Fluko Laboratory Equipment Co., Ltd., Watertown, MA, USA); 2500 gel imager and electrophoresis apparatus (Bio-Rad, Hercules, CA, USA); microplate reader (Thermo Fisher Instruments Co., Ltd., Waltham, MA, USA); quantitative polymerase chain reaction (qPCR) instrument (Applied Biosystems, Waltham, MA, USA); TRIzol reagent, SuperScript III reverse transcription (RT) kit, SYBR qPCR mix (ABI), terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) fluorescent staining kit (Roche, Basel, Switzerland) and loading buffer, protease inhibitor and bicinchoninic acid (BCA) protein concentration assay kit (Biosharp, Heifei, China).

Establishment of Animal Models

A total of 40 male healthy SD rats were habituated. They were fasted overnight before surgery, and were anesthetized with pentobarbital (40 mg/kg) and ventilated (50 times/min), followed by thoracotomy performed under sterile conditions. Next, a non-invasive suture was used to ligate the left coronary artery for 60 min of ischemia.

Then, the ligature was loosened for reperfusion for 2 h to establish rat models of myocardial I/R. Thereafter, myocardial I/R rats were divided into myocardial I/R model group (model group) and myocardial I/R model+UTI treatment group (treatment group, 5 mg/kg, intraperitoneal injection, once a day for one week). Additionally, 20 SD rats were selected as normal group. They were subjected to the same surgical procedures except for the ligation of the left coronary artery. This investigation was approved by the Animal Ethics Committee of China Medical University Animal Center.

Examination of Myocardial Function

Blood samples were routinely collected from femoral veins, let stand at room temperature for 20 min and centrifuged at 4°C and 2500 g for 10 min to separate and collect serum. Lastly, a biochemical analyzer was employed to determine changes in alanine aminotransferase (ALT), creatine kinase (CK), and creatinine (Scr).

Detection of Cardiac Physiological Function Indexes in Rats

7500 ultrasound machine (Philips Medical, Amsterdam, the Netherlands) and magnetic resonance imaging (MRI) and echocardiography (ECG, with a probe frequency of 10 MHz) systems were employed to detect the left ventricular function indicators. Left ventricular end-diastolic diameter (LVEDd), left ventricular end-systolic diameter (LVESd), ejection fraction (EF), and fractional shortening (FS) in rats of each group were recorded in a supine position.

Determination of Serum Inflammatory Cytokines in Rats

Blood was routinely sampled from femoral veins and centrifuged at 1500 g and 4°C for 30 min. After that, a pipette was used to extract the supernatant. Serum levels of tumor necrosis factor-alpha (TNF-α), IL-6, and matrix metalloproteinase-9 (MMP-9) were measured using corresponding ELISA kits based on the actual conditions and the instructions. The absorbance was detected using a microplate reader.

Determination of Stress Indicators in Myocardial Tissues of Rats

The rats were intraperitoneally anesthetized and then sacrificed. Next, left ventricular tissues (0.5 g) were homogenated on ice and centrifuged at 1200 g and 4°C for 30 min, and the supernatant was collected using the pipette. Thereafter, the levels of reactive oxygen species (ROS), superoxide dismutase (SOD, and malondialdehyde (MDA) were measured using corresponding ELISA kits in accordance with the actual conditions and the instructions. The absorbance in each group was read using the microplate reader.

Determination of Apoptosis Through Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining Assay

The apoptosis of myocardial cells was detected in prepared paraffin sections according to the instructions of the TUNEL apoptosis assay kit (Roche, Basel, Switzerland). Paraffin sections were first deparaffinized, washed with phosphate-buffer saline (PBS), and incubated in proteinase K working solution. After blockage, sections were fixed and infiltrated with 0.1% Triton X-100. Next, the TUNEL assay kit was used for fluorescein isothiocyanate (FITC) end labeling of the fragmented deoxyribonucleic acids (DNAs). The FITC-labeled TUNEL-positive cells were captured using a fluorescence microscope. A total of 5 fields of view were selected to calculate the number of TUNEL-positive cells.

Determination of Gene Expression Via Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total ribonucleic acids (RNAs) were extracted from rat myocardial tissues in each group with TRIzol reagent, and then reverse transcription was conducted to obtain complementary DNA (cDNA) strands after the purity and concentration of RNAs were qualified. Thereafter, primer amplification was performed using a 20 μL system (2 μL cDNA, 10 μL mix, 2 μL primer, and 6 μL ddH_2O, 40 cycles), followed by PCR amplification (pre-denaturation at 95°C for 2 min, and 40 cycles at 94°C for 20 s, 60°C for 20 s and 72°C for 30 s). The primer sequences (Table I) of target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (an internal reference) were designed based on the sequences on GenBank, and the expression levels of target genes were measured *via* qRT-PCR. The messenger RNA (mRNA) expressions in rat myocardial tissues in each group were calculated through $2^{-\Delta\Delta Ct}$.

Table I. PCR primer sequences.

Gene	Primer sequence
GAPDH	F: 5'-CAGTGCCAGCCTCGTCTCAT-3' R: 5'-AGGGCCATCCACAGTCTTC-3'
Glucose-regulated protein 78 (GRP78)	F: 5'-CTTCTAGGCATCCCTTCCTTACAGC-3' R: 5'-GAAGCGCTCACGAGACAGGTGGA-3'
Caspase-12	F: 5'-ATGCCTACCGCACCCGGTTACTAT-3' R: 5'-TACGTTCCGGTTAACACGAGTGAG-3'
B-cell lymphoma 2 (Bcl-2)	F: 5'-ATTGGTGCTCTTGAGATCTCTGG-3' R: 5'-TAACCATCGATCTTCAGAAGTCTC-3'

Western Blot

The rat heart tissues were cut into pieces and homogenated in radio immunoprecipitation assay (RIPA) lysis buffer at a ratio of 100 mg to 1 mL. Next, extracted proteins were quantified using the bicinchoninic acid (BCA) protein assay kit. Protein samples were subjected to electrophoresis and loaded on PVDF membranes. After membranes were blocked, they were incubated with primary antibody overnight and then secondary antibody for 1 h. Freshly prepared enhanced chemiluminescence (ECL) mixture was applied for band exposure in a dark place using an Odyssey membrane scanner. GAPDH was used as the internal reference. Western blotting bands were quantified using Image Lab (Media Cybernetics, Silver Springs, MD, USA) software.

Statistical Analysis

All experimental data were processed by Statistical Product and Service Solutions (SPSS) 21.0 (SPSS, Chicago, IL, USA) software and subjected to multiple comparisons. The experimental results obtained were expressed as mean ± standard deviation ($\bar{x} \pm$ SD), and $p<0.05$ suggested that the difference was statistically significant. GraphPad Prism 8.0 (La Jolla, CA, USA) was used to plot histograms.

Results

Determination of Myocardial Function Indicators

Relative levels of ALT, CK, and Scr were significantly higher in model group than those in normal group ($p<0.05$), while they were evidently reduced in treatment group ($p<0.05$). Increased myocardial function indexes verified the successful construction of the myocardial I/R model. Moreover, UTI exerted certain protective effects on rats with myocardial I/R injuries (Table II).

Determination of Cardiac Function Indexes

Rats in model group had notably lowered FS and EF, but markedly elevated LVEDd and LVESd in comparison with normal group ($p<0.05$). UTI treatment partially reversed the above trends ($p<0.05$) (Table III).

Determination of Cytokines

The levels of the inflammatory factors TNF-α, IL-6, and MMP-9 were remarkably higher in model group than those in the other two groups. Meanwhile, their levels were lower in treatment group compared with those in model group ($p<0.05$), suggesting that massive inflammatory factors produced following myocardial I/R could be improved by UTI (Table IV).

Table II. Serum myocardial biochemical indicators detected.

Group	ALT (U/L)	CK (U/L)	Scr (Umol/L)
Normal group	42.6 ± 0.9	68.1 ± 1.5	14.7 ± 2.3
Model group	120.1 ± 0.6[a]	181.7 ± 0.9[a]	78.9 ± 1.2[a]
Treatment group	60.3 ± 0.7[b]	80.2 ± 0.7[b]	23.4 ± 4.7[b]

Note: The ALT, CK, and Scr levels are higher in the model group than those in the normal group, while they clearly decline in the treatment group, implying that myocardial function indicators are abnormal. [a]$p<0.05$ *vs*. normal group, [b]$p<0.05$ *vs*. model group.

Table III. Cardiac function indexes in rats determined by MRI and ECG.

Group	LVEDd (mm)	LVESd (mm)	EF (%)	FS (%)
Normal group	3.1 ± 0.2	4.1 ± 0.4	68.6 ± 3.7	56.4 ± 3.5
Model group	8.9 ± 0.5[a]	7.8 ± 0.2[a]	44.7 ± 3.4[a]	37.8 ± 2.1[a]
Treatment group	5.0 ± 0.1[b]	5.5 ± 0.3[b]	61.8 ± 2.5[b]	50.8 ± 1.2[b]

Note: The FS and EF are lower in the model group than those in the normal group, while the LVEDd and LVESd exhibit contrary tendencies. [a]$p<0.05$ *vs*. normal group, [b]$p<0.05$ *vs*. model group.

Table IV. Levels of inflammatory factors.

Group	IL-6 (mg/L)	TNF-α (fmol/mL)	MMP-9 (ng/mL)
Normal group	51.8 ± 5.3	38.4 ± 5.3	84.1 ± 4.1
Model group	176.5 ± 5.1[a]	88.8 ± 6.9[a]	302.2 ± 5.3[a]
Treatment group	71.4 ± 4.7[b]	49.8 ± 3.4[b]	103.2 ± 1.8[b]

Note: The levels of TNF-α, IL-6 and MMP-9 are distinctly raised in the model group, but significantly reduced in the treatment group. [a]$p<0.05$ *vs*. normal group, [b]$p<0.05$ *vs*. model group.

Contents of SOD, MDA, and ROS

As shown in Table V, the contents of MDA and ROS in myocardial tissues were elevated in model group ($p<0.05$), and markedly reduced in treatment group ($p<0.05$), while the SOD content was the contrary ($p<0.05$).

Myocardial Apoptosis Level in Rats Determined Through TUNEL Staining Assay

TUNEL staining assay was conducted to determine the apoptosis level in rat myocardial tissues in each group. It is shown that there were almost no apoptotic myocardial cells in normal group, and a large number of apoptotic myocardial cells in model group. Myocardial apoptosis was remarkably attenuated in treatment group (Figure 1).

Expressions of ESR and Apoptosis-related Genes

The results of qRT-PCR assay shown in Figure 2 revealed that treatment group exhibited significantly decreased expression levels of Caspase-12 and GRP78, and upregulated Bcl-2 ($p<0.05$). Rats in treatment group had overtly increased expression levels of Caspase-12 and GRP78, and a clearly lowered expression level of Bcl-2 ($p<0.05$), suggesting that the treatment with UTI inhibited the occurrence of ERS and apoptosis in myocardial tissues. In addition, protein expression changes of Caspase-12, GRP78, and Bcl-2 were identical to their mRNA levels (Figure 3).

Discussion

Ischemic heart disease is the leading cause of death and disability all over the world. Timely reperfusion therapy is a major treatment option for IHD, but reperfusion itself will cause severe heart injury, that is, myocardial (I/R) injury[19]. The ER is a multifunctional organelle participating in protein synthesis and folding in eukaryotic cells. I/R injury could lead to the accumulation of unfolded

Table V. Content of SOD, MDA, and ROS in myocardial tissues.

Group	SOD (U/mg)	MDA (mmol/g)	ROS (U/L)
Normal group	49.5 ± 0.9	2.9 ± 1.1	6.1 ± 0.5
Model group	22.8 ± 0.6a	19.2 ± 0.8[a]	38.2 ± 0.8[a]
Treatment group	41.2 ± 0.7[b]	5.1 ± 0.4[b]	10.4 ± 0.6[b]

Note: The content of MDA and ROS rises in the model group ($p<0.05$) and dramatically declines in the treatment group ($p<0.05$), while the SOD content is the contrary ($p<0.05$). [a]$p<0.05$ *vs*. normal group, [b]$p<0.05$ *vs*. model group.

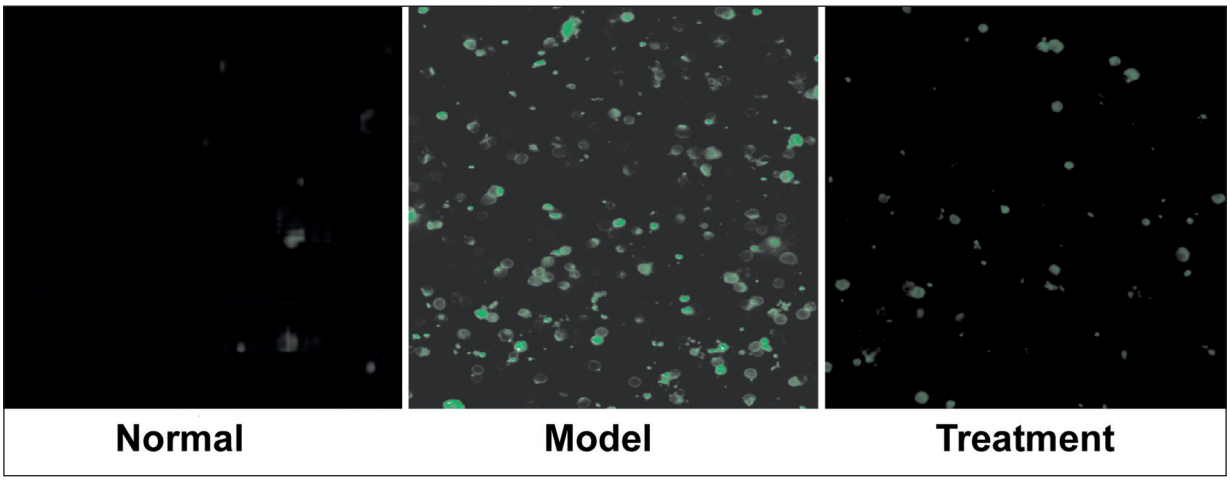

Figure 1. Apoptosis determined through TUNEL staining assay. The number of apoptotic myocardial cells is elevated in model group and reduced in treatment group (magnification 100×).

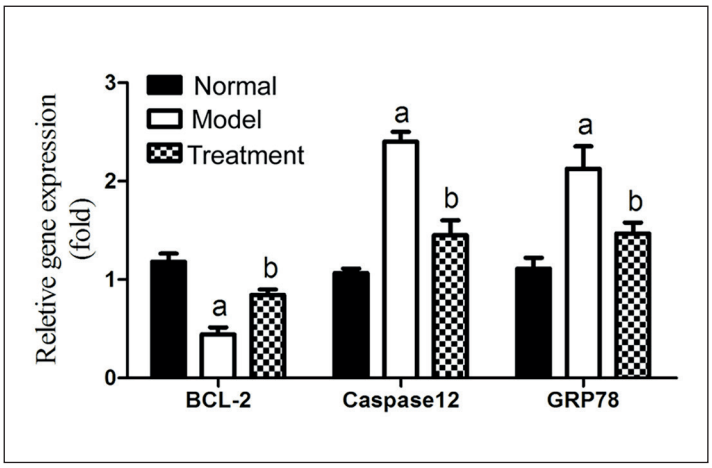

Figure 2. The mRNA expression levels of Casepase-12, GRP78, and Bcl-2. The expression levels of Caspase-12 and GRP78 are reduced in treatment group and raised in model group, while the expression level of Bcl-2 is elevated in treatment group and declines in model group. [a]$p<0.05$ *vs*. normal group, [b]$p<0.05$ *vs*. model group.

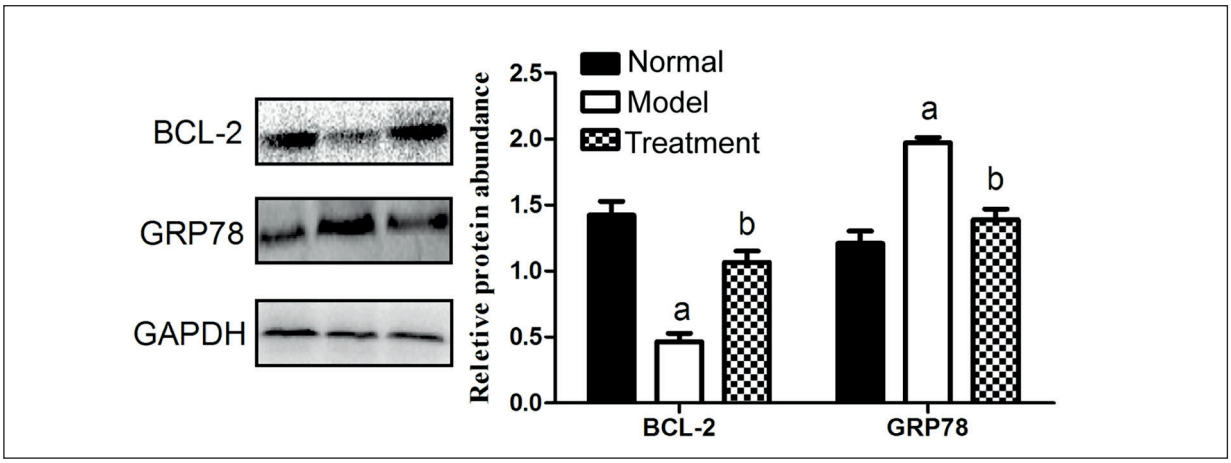

Figure 3. Protein expression levels of Casepase-12, GRP78, and Bcl-2. Bcl-2 expression level is remarkably elevated in treatment group and clearly reduced in model group, while GRP78 protein expression level is obviously down-regulated in treatment group and evidently increased in model group ($p<0.05$). [a]$p<0.05$ *vs*. normal group, [b]$p<0.05$ *vs*. model group.

proteins in the ER, inducing a severe UPR. Persistent UPR ultimately leads to apoptosis[20]. Hence, reducing excessive UPR, namely ERS, is important for alleviating I/R injury. In this study, it was discovered that the FS and EF in model group were evidently lower than those in normal group, and LVEDd and LVESd in model group were clearly higher than those in normal group, suggesting that the rat model of myocardial I/R is successfully constructed. Besides, serum levels of ALT, CK, and Scr were remarkably higher in model group than those in normal group, and they were markedly reduced in treatment group. It is suggested that myocardial function indexes were elevated in the development and progression of ERS-induced apoptosis during myocardial I/R, offering an important reference for early diagnosis. In addition, the levels of inflammatory factors TNF-α, IL-6, and MMP-9 were measured. Their levels were notably higher in model group than those in the other two groups, while they significantly declined in treatment group, indicating that inflammatory factors produced during ERS-induced apoptosis further aggravated myocardial I/R. The above results were consistent to a previous study[21].

I/R injury facilitates ERS. Increasing evidence has suggested that ROS plays a crucial role in I/R-induced cell injury or death. It is recently reported that ERS-mediated apoptosis is involved in the pathogenesis of various diseases such as diabetes, neurodegenerative diseases, and I/R[22]. ERS refers to the dysfunction of ER due to oxidative stress, hypoxia-ischemia injury, disordered calcium homeostasis, and misfolding of highly expressed proteins. The typical feature of ERS is GRP78 up-regulation[23]. This study found that the content of the oxidative stress indicators MDA and ROS in myocardial tissues increased in model group and distinctly decreased in treatment group, while the SOD expression was lowered in model group and raised in treatment group. Hence, it is believed that oxidative stress occurred during myocardial I/R. Apoptosis is capable of maintaining cell stability, eliminating harmful substances in cells, and quickly initiating apoptosis once being stimulated. Apoptosis-related genes and proteins are responsible for regulating apoptosis, including Bcl-2, Bax, and Caspase-12[24]. In this study, the apoptosis in rat myocardial tissues in each group was detected by TUNEL staining assay. The results revealed that there was almost no myocardial apoptosis in normal group, and a large number of apoptotic myocardial cells in model group. However, apoptosis in treatment group was relieved after treatment with UTI. Moreover, both mRNA and protein expression levels of Caspase-12 and GRP78 evidently decreased, and the expression level of Bcl-2 significantly increased in treatment group, while the above expression levels exhibited opposite tendencies in model group. It is indicated that the treatment with UTI inhibits the occurrence of ERS and apoptosis in myocardial tissues. These results were consistent with previous studies[25,26]. The above results of this research proved the protective effect of UTI against myocardial I/R injury in rats by suppressing ERS-induced apoptosis.

Conclusions

In summary, UTI may have a protective effect against myocardial I/R injury mainly through repression of ERS-induced apoptosis, which alleviates inflammatory cell infiltration and oxidative stress damage. Our results provide a theoretical basis for the prevention and treatment of myocardial I/R injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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